Insect-Mediated Microevolution of Flowering Plants

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Zusammenfassung

Die Essenz der Evolutionsbiologie ist es die Prozesse zu verstehen, welche zur enormen Vielfalt an Organismen geführt haben. Für die alten Naturalisten war es schlicht undenkbar, dass in Zeitspannen jenseits unserer Vorstellung kleine, graduelle Veränderungen Lebensformen hervorbringen können, welche sich so grundlegend unterscheiden wie zum Beispiel eine Maus und ein Baum. Inzwischen ist es jedoch allgemein anerkannt, dass solche mikro-evolutionären Veränderungen innerhalb organismischer Populationen die Grundlage für die grossen makro-evolutionären Wandlungen über dem Artniveau sind.

Die Gruppe der Blütenpflanzen (Angiospermen) zeichnet sich durch besonders viele und diverse mikro-evolutionäre Veränderungen aus. Ungleich anderen Pflanzengruppen sind Angiospermen zum Austausch ihrer Keimzellen auf Interaktionen mit Bestäubern angewiesen welche hauptsächlich zu den Insekten gehören. Nicht alle biotischen Interaktionen sind jedoch von Vorteil für die Pflanze, und Angiospermen müssen gleichzeitig die Anlockung mutualistischer Bestäuberinsekten und die Abschreckung antagonistischer Insekten wie zum Beispiel Herbivoren maximieren. Die Kommunikation mit Insekten beeinflusst somit sowohl die Reproduktion als auch das Überleben von Pflanzen, was die Entstehung mikro-evolutionärer Veränderungen in Pflanzenmerkmalen fördert.

Das ungebrochene Interesse an Insekten-induzierter Mikroevolution von Pflanzen hat unsere Kenntnisse über die ihr zugrunde liegenden Mechanismen beachtlich vorangebracht. Inzwischen haben wir eine ziemlich klare Vorstellung davon wie Mutationen im Erbgut entstehen, wie die Ausprägung phänotypischer Merkmale durch Mutation beeinflusst werden, und wie Veränderungen im Phänotyp Insekten-induzierte Selektionsdrücke verändern können. Der grösste Teil dieser Kenntnisse stammt jedoch von theoretischen Modellierungen oder empirischen Studien mit Modellorganismen, während Untersuchungen in natürlichen Systemen häufig lückenhaft sind. In Konsequenz konnten viele klassische, gut etablierte genetische Hypothesen bis jetzt noch nie eindeutig in der Natur bestätigt werden. Besonders wenig Evidenz ist vorhanden für das altbekannte und einst sehr populäre Konzept der Überdominanz, eine Form von balancierter Selektion welche die an einem Genort heterozygoten Individuen den homozygoten Individuen bevorteilt, wodurch genetische und phänotypische Variation in Mikroevolution verharren. Die Erforschung der Grundkonzepte der Mikroevolution bleibt jedoch nicht stehen, und neue Erkenntnisse führen zur Formulierung von neuen Hypothesen. Die vor kurzem entdeckten epigenetischen Effekte, durch welche biotische Interaktionen erbliche Veränderungen in der Funktion von Genen hervorrufen können ohne die Erbgutsequenz zu ändern, macht eine Überdenkung unseres Konzeptes der Vererbungslehre vonnöten. Die aktuelle Forschung muss deshalb gleichzeitig alte Hypothesen verifizieren sowie neue Hypothesen ins Grundgerüst der Mikroevolution einpassen.

Ziel der vorliegenden Arbeit ist, sowohl klassische als auch neue mikro-evolutionäre Hypothesen zu überprüfen. Erstens versucht sie die Bedeutung epigenetischer Veränderungen in der Wechselwirkung zwischen Pflanzen und Insekten zu beleuchten. Zweitens ist sie bestrebt das Vorkommen insekten-induzierter Überdominanz in natürlichen Pflanzenpopulationen nachzuweisen. Kapitel I und II beinhalten den epigenetischen Teil, und Kapitel III beinhaltet den

genetischen Teil dieser Arbeit über insekteninduzierte Mikroevolution in Pflanzen.

In Kapitel I wurde die Nutzpflanze *Brassica rapa* als Modellorganismus verwendet um den Einfluss von Herbivorie auf Blütenmerkmale und DNS-Methylierung zu untersuchen. DNS-Methylierung ist eine epigenetische Codierung welche mit der Regulation von Genen assoziiert ist. In einem ersten Schritt wurden *B. rapa* Pflanzen Blattschäden durch *Pieris brassicae* Raupen zugefügt, und die dadurch induzierten Änderungen in der DNS-Methylierung mit methylierungssensitiven AFLPs (MSAP) analysiert. Diese Analyse hat gezeigt, dass Herbivorie zu genomweiter Demethylierung sowohl in Blättern als auch in Blüten von *B. rapa* geführt hat, was mit einer Reduktion in der Anzahl, Form und Duft der Blüten einherging. Ein Vergleich mit Pflanzen, in welchen anti-herbivore Reaktionen mit dem Pflanzenhormon Jasmonsäure künstlich herbeigeführt wurden hat ähnliche Demethylierungsmuster in Blätter aufgezeigt. Sowohl die Methylierung als auch die Form der Blüten dieser Pflanzen haben sich jedoch von den *P. brassicae*-infizierten Pflanzen unterschieden. In einem zweiten Schritt wurden die Genome von fünf verschiedenen *B. rapa* Geschwistergruppen mit 5-Azacytidin, einem chemischen Inhibitor von DNS-Methyltransferasen, auf standardisierte Art und Weise demethyliert. Dies hat zu einer genotyp-spezifischen Änderung in der Morphologie und im Duft der Blüten geführt, wodurch die Attraktivität der Pflanzen auf den Bestäuber *Bombus terrestris* signifikant verringert wurde. Insgesamt deuten diese Resultate darauf hin, dass DNS-Methylierung eine wichtige Rolle in der Anpassung von Blütensignalen an Änderungen in der Insektenvielfalt spielt.

In Kapitel II wurde die Entstehung und Vererbung Herbivorie-induzierter Veränderungen in *Brassica rapa,* sowie deren Effekt auf Interaktionen mit Insekten untersucht. Zwei Generationen abstammend von einer einzelnen, aus einer Inzuchtlinie stammenden *B. rapa* Pflanze wurden Blattschäden durch den generalistischen Herbivoren *Mamestra brassicae* sowie den spezialisierten Herbivoren *Pieris brassicae* zugefügt. Innerhalb dieser zwei Pflanzengenerationen wurden Herbivorie-induzierte Veränderungen sowohl in der Morphologie und in reproduktiven Merkmalen, als auch in der Emission von Blüten, und Blattduftstoffen festgestellt. Des Weiteren haben Bioassays gezeigt, dass durch Herbivorie beschädigte Pflanzen einerseits attraktiver für parasitoide Wespen der Art *Cotesia glomerata* sind, andererseits jedoch weniger vom Bestäuber *Bombus terrestris* besucht werden, was auf einen möglichen trade-off hinweist. Nach zwei Pflanzengenerationen wurde die Behandlung mit Herbivoren gestoppt, und die erwähnten Merkmale für weitere zwei Generationen untersucht um die Beibehaltung der induzierten Veränderungen zu dokumentieren. Während Änderungen in Blatt-, und Blütenduftstoffen in der ersten Herbivorie-freien Generation verschwanden, waren einige Veränderungen in der Morphologie und in reproduktiven Merkmalen der Pflanzen in der zweiten Herbivorie-freien Generation nach wie vor messbar. Weder Parasitoide noch Bestäuber machten jedoch einen Unterschied zwischen den Pflanzengruppen mit unterschiedlicher Behandlung in der Vergangenheit. Diese Resultate weisen darauf hin, dass die Vererbung Herbivorie-induzierter Veränderungen bevorzugt in Merkmalen mit begrenzten Ressourcen stattfindet, welche mit dem Wachstum und der Vermehrung der Pflanzen einhergehen. Das Fehlen von Änderungen in Interaktionen zwischen Pflanzen und Insekten ist

jedoch auf die Kurzlebigkeit der Änderungen in Blatt-, und Blütenduftstoffen zurückzuführen.

In Kapitel III wurde eine Kombination von ökologischen, evolutionären und entwicklungsbiologischen Ansätzen (Eco-Evo-Devo) verwendet um die Entstehung eines Polymorphismus in der Blütenfarbe der alpinen Orchidee *Gymnadenia* (*Nigritella*) *rhellicani* zu untersuchen. In ihrem gesamten alpinen Habitat produziert *G: rhellicani* kompakte Infloreszenzen aus nektarhaltigen, nach Schokolade duftenden Blüten mit dunkelroten bis schwarzen Petalen. In einer bestimmten Population auf der Seiser Alm im Südtirol in Italien ist die Blütenfarbe jedoch hochgradig polymorph mit 62% wildtyp-schwarz, 28% hellrot, und 10% weissblütigen Individuen. Daten zur Veränderung der relativen Häufigkeit der Farbmorphe über die letzten zwanzig Jahre weisen darauf hin, dass die Blütenfarbe unter Überdominanz stehen könnte. Messungen haben gezeigt, dass (1) sich diese Farbmorphe in anderen Blütenmerkmalen wie Anzahl, Form, Temperatur und Duft der Blüten nicht unterscheiden, und (2) *G. rhellicani* eine diploide Pflanzenart ist, welche zur Reproduktion auf Insekten angewiesen ist. In einem nächsten Schritt wurden Insektenbesuche mit Zeitrafferkameras festgehalten, was gezeigt hat, dass Bienen und Fliegen als Bestäuber gegensätzlich-direktionale Selektion auf die Blütenfarbe ausüben, wodurch die Samenproduktion im intermediären Morph maximiert wird. Als Nächstes wurden molekulare Untersuchungen durchgeführt um die genetische Basis dieses Polymorphismus zu verstehen: (1) Eine Kombination aus phänotypischen, metabolomischen und transkriptomischen Analysen hat gezeigt, dass sich die Farbmorphe lediglich in der Konzentration eines Anthocyanfarbstoffes sowie zwei Derivaten hiervon unterscheiden. Diese Konzentrationsunterschiede korrelieren mit Expressionunterschieden eines *Anthocyanidin Synthase* (*ANS*) Genes. (2) Mittels einer transkriptomweite Assoziationskartierung (TWAS) wurde ein Einzelnukleotid-Polymorphismus (SNP) identifiziert, welcher im intermediären Morph heterozygot vorliegt und ein vorzeitiges Stop-Codon in einem *ANS* regulierenden *R2R3-MYB* Transkriptionsfaktor einführt. (3) Auf dem Monte Bondone im Trentino in Italien wurde eine zweite farbpolymorphe *G. rhellicani* Population lokalisiert, welche einen Phänotyp analog zur Population auf der Seiser Alm aufweist. Mittels Genotypisierung wurde entdeckt, dass diese Population eine Leserastermutation im selben *R2R3-MYB* Transkriptionsfaktor aufweist, was auf eine parallele Evolution von hellen Blütenfarben in *G. rhellicani* hindeutet. Insgesamt weisen diese Entdeckungen darauf hin, dass Polymorphismen in Blütenfarben durch Bestäuber-induzierte Überdominanz evolvieren können.

In Summe bieten die Resultate dieser Arbeit empirischen Nachweis, dass sowohl die klassische genetische Hypothese der Überdominanz als auch die neue Hypothese der epigenetischen Vererbung eine Rolle in Insekten-induzierter Mikroevolution spielen. Der epigenetische Teil zeigt, dass pflanzenfressende Insekten Veränderungen in der DNS-Methylierung hervorrufen können, welche von erblichen phänotypischen Veränderungen begleitet sind, welche wiederum Interaktionen von Pflanzen mit bestäubenden und parasitoiden Insekten beeinflussen können. Es besteht jedoch kein Hinweis darauf, dass adaptive induzierte phänotypische Veränderungen spezifisch oder bevorzugt vererbt werden, und die breite Wirkung von Herbivorie auf die Methylierung stellt die generelle Wichtigkeit einzelner induzierter epigenetischer Veränderungen in

langwierigen makro-evolutionären Prozessen infrage. Der genetische Teil zeigt, dass sich Bestäuber Blütenpolymorphismen durch Überdominanz erhalten können, vor Allem wenn die Pflanzenart diploid, nektarhaltig und weder autogam noch apomiktisch ist, die verantwortliche Mutation sich in einem optimal pleiotropen Element befindet, die Allele co-dominant sind, und das polymorphe Blütenmerkmal stark fitness-relevant ist. Die ultimativen Gründe für die Entstehung von Überdominanz können jedoch nur verstanden werden wenn die evolutionäre Vergangenheit einer Art miteinbezogen wird. Abschliessend sei erwähnt, dass die Resultate dieser Arbeit nicht nur die vielfältigen Wege beleuchten, auf welchen die unablässige Interaktion mit Insekten immer neue Variation an Blütenformen hervorbringt Sie betonen auch, dass ein Verständnis der grossen Prozesse in der Natur nur durch ein permanentes Verknüpfen und Neuverknüpfen aller ihnen zugrunde liegenden kleinen Bruchstücke entstehen kann.

Synopsis

Understanding the mechanisms behind the enormous diversity of organisms is the essence of evolutionary biology. The thought that in timespans beyond our imagination, small, gradual changes could give rise to life forms as different as for example a mouse and a maple tree was inconceivable for early naturalists. Meanwhile, it is universally acknowledged that micro-evolutionary changes within populations of organisms provide the raw material for the great macro-evolutionary transitions beyond the species level.
A particularly diverse accumulation of micro-evolutionary changes can be found in flowering plants (angiosperms). Unlike other plant groups, angiosperms rely on specific interactions with (mainly insect) pollinators for the transfer of gametes. However, not all biotic interactions are beneficial for the plant, and angiosperms have to simultaneously maximize the attraction of mutualistic pollinator insects and the deterrence of antagonistic insects such as herbivores. Plant-insect communication thus affects both plant reproduction and survival, propelling micro-evolutionary changes of plant traits.

The unabated interest in insect-mediated plant microevolution has led to a considerable advance in our understanding of the underlying mechanisms. We now have a fairly clear picture of how DNA mutations can arise, how mutations affect the phenotypic expression of plant traits, and how phenotypic trait changes can alter selective pressures imposed by insects. Nevertheless, most of this information is based on theoretical models and empirical studies on model systems, and investigations in natural systems are often fragmentary. As a consequence, many classic, well-established genetic hypotheses have never been unequivocally verified in nature. Particularly little evidence is available for the long-lasting and once popular hypothesis on the action of overdominance, a form of balancing selection favouring heterozygotes at a single locus over both homozygotes, which forces genetic and phenotypic variation to persist in microevolution. At the same time, research on the basis of microevolution continues, and new findings lead to the formulation of new hypotheses. The recent discovery of epigenetic effects, by which biotic interactions can induce heritable alterations in gene function without any DNA sequence change, requires a rethinking of our concept of inheritance. Current research thus has to simultaneously verify old, and integrate new concepts in the micro-evolutionary framework.

The aim of the present work is to test both classic and novel micro-evolutionary hypotheses: Firstly, it aims at elucidating the importance of epigenetic changes in the evolution of plant interactions with pollinators and herbivores. Secondly, it aims at confirming the occurrence of pollinator-mediated overdominance in natural plant populations. Chapter I and II contain the epigenetic part, and chapter III contains the genetic part of this work on insect-mediated plant microevolution.

In chapter I, the model crop plant *Brassica rapa* was used to assess the impact of foliar herbivory on floral traits and DNA methylation, an epigenetic encoding associated with regulation of gene transcription. In a first step, *B. rapa* plants were subjected to leaf damage by the caterpillar *Pieris brassicae,* and resulting DNA methylation changes were detected with methylation-sensitive amplified fragment length polymorphism (MSAP). This analysis showed that

herbivory was associated with genome-wide demethylation in both leaves and flowers of *B. rapa* as well as a reduction in flower number, morphology and scent. A comparison to plants in which anti-herbivore reactions were artificially induced with the defence hormone jasmonic acid showed similar demethylation patterns in leaves. However, both the floral methylome and phenotype differed significantly from *P. brassicae* infested plants. In a second step, genomes of five different *B. rapa* full-sibling groups were subjected to standardized de-methylation with 5-azacytidine, a chemical inhibitor of DNA methyltransferases. This resulted in a genotype-specific change of floral morphology and scent, which significantly reduced the attractiveness of the plants to the pollinator bee *Bombus terrestris.* Overall, these results suggest that DNA methylation plays an important role in adjusting plant signalling in response to changing insect communities.

In chapter II, the emergence and transmission of herbivory-induced changes, and their impact on interactions with insects were assessed in *Brassica rapa.* Two plant generations descending from a single, highly inbred, and rapid-cycling *B. rapa* plant were subjected to leaf herbivory by the generalist caterpillar *Mamestra brassicae* and the specialist *Pieris brassicae.* Within two plant generations, herbivory induced changes in plant morphology and reproductive traits as well as in flower- and leaf volatile emission. Also, bioassays showed an increased attractiveness of herbivore-damaged plants to the parasitoid wasp *Cotesia glomerata,* as well as a decreased visitation by the pollinator *Bombus terrestris,* which indicates a potential trade-off. After two plant generations, herbivory treatment was ceased and trait values were recorded for two subsequent generations without herbivory to assess the retention of the induced trait changes. While changes in floral- and leaf volatiles disappeared in the first generation after herbivory, some changes in morphology and reproductive traits were still measurable two generations after herbivory. However, neither parasitoids nor pollinators further discriminated between groups with different past treatments. These results suggest that transmission of herbivore-induced changes occurs preferentially in resource-limited traits connected to plant growth and reproduction. However, the lack of alterations in plant-insect interactions is likely due to the transient nature of volatile changes.

In chapter III, a combination of ecological and evolutionary-developmental approaches (eco-evo-devo) was applied to assess the emergence of a floral colour polymorphism in the Alpine orchid *Gymnadenia* (*Nigritella*) *rhellicani.* Throughout its Alpine habitat, *G. rhellicani* produces dense inflorescences of rewarding (nectar containing), chocolate-scented flowers with dark-red to almost black petals. However, one particular population located on Seiser Alm, South Tyrol, Italy, is highly polymorphic with 62% wild-type black, 28% bright red, and 10% white individuals. Data on the change of relative morph abundances over twenty years suggest that floral colour could be under overdominant selection. Measurements revealed that (1) the colour morphs do not differ in other floral traits such as flower number, shape, temperature, and scent, and (2) *G. rhellicani* is diploid and depends on pollinators for reproduction. In a next step, pollinator visits were recorded with time-lapse cameras, which showed that bee and fly pollinators exert opposite directional selection on flower colour, maximising seed set in the intermediate morph. Next, molecular investigations were performed to uncover the genetic basis of the

polymorphism: (1) A combination of phenotypic, metabolomic, and transcriptomic analyses showed that the morphs differ solely in the concentration of one anthocyanin pigment and two derivatives, which is linked to differential expression of an *anthocyanidin synthase* (*ANS*) gene. (2) Transcriptome-wide association mapping (TWAS) identified a single-nucleotide polymorphism (SNP) heterozygous in the intermediate morph, which introduces a premature stop codon in an *ANS* regulating *R2R3-MYB* transcription factor. (3) Genotyping of a second colour polymorphic *G. rhellicani* population on Monte Bondone, Trento, Italy, with a phenotype analogous to the one on Seiser Alm revealed a single-nucleotide deletion in the same *R2R3-MYB* transcription factor, suggesting parallel evolution of bright floral colours in *G. rhellicani.* Altogether, these findings indicate that floral colour polymorphisms can evolve by pollinator-mediated overdominant selection.

In summary, the results of this work provide empirical evidence for a role of both the classic genetic concept of overdominance and the novel concept of epigenetic inheritance in insect-mediated plant microevolution. The epigenetic part shows that insect herbivores can induce DNA methylome changes accompanied by heritable phenotypic alterations, which in turn can influence plant interactions with pollinator and parasitoid insects. However, there is no indication that adaptive induced phenotypic changes are specifically or preferentially transmitted, and the broad impact of herbivory on the methylome questions the general importance of single induced epigenetic changes in long-term macro-evolutionary processes. The genetic part shows that pollinators can maintain floral polymorphisms through overdominance, preferentially if the plant is diploid, rewarding, and neither autogamous nor apomictic, the responsible mutation resides in an optimal pleiotropic element, the alleles are codominant, and the polymorphic trait is highly fitness relevant. However, the ultimate reasons for the emergence of overdominant selection may only be understood in consideration of the evolutionary history of a species. To conclude, this work not only highlights the manifold ways, in which perpetual interactions with insects lead to endless variation in floral forms, it also emphasizes that an understanding of the grand processes in nature only arises through perpetual assembly and re-assembly of all their underlying bits and pieces.

General Introduction

Microevolution - evolution on the species level

"*The production of form from formlessness in the egg-derived individual, the multiplication of parts and the orderly creation of diversity among them, in an actual evolution, of which anyone may ascertain the facts, but of which no one has dissipated the mystery in any significant measure. This* ***microevolution*** *forms an integral part of the grand evolution problem and lies at the base of it, so that we shall have to understand the minor process before we can thoroughly comprehend the more general one*."

Robert Greenleaf Lewitt, 1909

The overwhelming diversity of life forms on Earth has fascinated Man throughout all time. Eager to grasp the origin and trajectories of life itself, biologists have long attempted to discern the order behind the apparent chaos: In 1735, Carl Linnaeus established the foundation of the present zoological and botanical nomenclature systems in his *Systema Naturae*, which hierarchically categorizes organisms according to their similarity (Linné, 1735). Through scientific progress especially in the field of genetics, the basal unit of this taxonomic system, the *species*, has been continuously redefined. This has led to the formulation of several different species concepts, which are under ongoing debate (De Queiroz, 1998). One widely accepted definition of species is the biological species concept, postulated in 1942 by Ernst Mayr: "*Species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups*" (Mayr, 1942).

Species are not stable entities, and one of the most central goals of evolutionary biology is to understand the processes by which species turn and sunder into novel species or higher taxa (Sobel *et al.*, 2010). Hypotheses on the action of evolutionary processes date back to the ancient Greeks (Mayr, 1982), and at the time Charles Darwin and Alfred Russell Wallace published their theory on evolution by natural selection (Darwin & Wallace, 1858; Darwin, 1859), several other concepts were in circulation as well. The rediscovery of Gregor Mendel's laws of inheritance in the year 1900 (Bateson & Mendel, 2013) not only provided the genetic basis which championed the theory of natural selection over all other concepts; it also split its supporters in two camps: On one hand, geneticists conducing laboratory experiments with model species such as *Drosophila* suggested that species accumulate many small-effect mutations which change them gradually over time. On the other hand, observations in the field convinced naturalists that new species only emerge through single large-effect mutations (Mayr & Provine, 1998).

In 1937, evolutionary biologist Theodosius Dobzhansky published his book *Genetics and the Origin of Species* (Dobzhansky, 1937), which reconciled

both geneticists and naturalists and became an essential part of the modern synthesis. Through the application of theoretical evolutionary concepts to case studies in natural populations, Dobzhansky showed that changes in allele frequencies at the species level, *i.e. microevolution*, can lead to the evolution of phylogenetic clades above the species level, *i.e. macroevolution*. Returning to the biological species concept, individuals could thus accumulate mutations, which, unless they are lost again, may increase in frequency within the population. This can either result in intraspecific polymorphisms within the population itself, or, in case the mutations become fixed, in intraspecific divergence between populations. In both cases, phenotypic differences may at one point prevent interbreeding between morphs, leading to the formation of reproductive barriers and the evolution of new species. Given enough time, microevolution can thus seamlessly transform into macroevolution, or in the words of Ernst Mayr: "*Transspecific evolution is nothing but an extrapolation and magnification of the events that take place within populations and species* [...] *it is misleading to make a distinction between the causes of micro- and macroevolution*" (Mayr, 1966).

Even after the modern synthesis, the debate over the connection between micro- and macroevolution has not come to a complete halt. Some scientists argue that the transition to a new species may require additional biological filters such as environmental influences (Erwin, 2000; Reznick & Ricklefs, 2009). Also, a few cases have been identified where macroevolution can occur spontaneously and without a gradual accumulation of mutations. Examples are whole-genome duplication (Taylor *et al.*, 2001; Ainouche & Wendel, 2014), the mutation of major developmental genes (Carroll, 2000), horizontal gene transfer (Lawrence, 2002), and probably endosymbiosis (Kutschera & Niklas, 2005). However, there seems to be a consensus that the majority of macroevolution can essentially be broken down into gradual micro-evolutionary processes (Carroll, 2001). Knowledge on the mode and action of microevolution, gained from both laboratory and field studies, is thus essential for an understanding of the great diversity of life.

Plant microevolution and the role of insect visitors

With an estimated 352000 species (The Plant List, 2017), flowering plants (angiosperms) are one of the most diverse organismic groups on Earth. Angiosperms have evolved in the early Cretaceous (Hickey & Doyle, 1977) and are thus much younger than other vascular plants such as ferns and gymnosperms, which originate from the late Devonian and late Carboniferous respectively (Pryer *et al.*, 2004; Wang & Ran, 2014). However, since their relatively recent appearance, the number of angiosperm species has exploded in a series of tremendous radiation events, and by today angiosperms dominate most of the plant biomes on this planet (Davies *et al.*, 2004).

Whilst the high species number of angiosperms remained elusive for early biologists (Darwin & Seward, 1903), it is now established that interactions with animals are an important factor behind this diversity (Waser & Ollerton, 2006; Kay & Sargent, 2009; Armbruster, 2014). Plants are sessile organisms and thus rely on external vectors for the transfer of gametes. Most gymnosperms employ abiotic agents such as wind or water for this transfer (Whitehead, 1969), which is not only an unspecific, but also a wasteful way of dispersal as only a minute fraction of pollen may reach a stigma in the end. As a compensation, pollen is usually produced in large quantities, and an individual may still (co)pollinate a large part of the surrounding conspecifics in a population (Faegri & Van der Pijl, 1979). Pre-pollination selection pressures in abiotic systems are thus mainly imposed on physical properties of flowers such as morphological adaptations to different fluid dynamics (Ackerman, 2000), resulting in a comparatively limited scope for micro-evolutionary changes of floral traits.

In contrast, a large part of the angiosperms relies on biotic agents such as insects for pollination, which decreases pollen waste and increases transfer specificity through targeted pollen dispersal (Faegri & Van der Pijl, 1979). However, unlike abiotic agents, insects are not present *per se* and only approach a flower if it increases their own fitness, *i.e.* their survival or reproductive success. Flowers of insect-pollinated plants thus usually contain a fitness-linked reward as well as signals to advertise the presence of this reward to the pollinator (Harder *et al.*, 2001). Rewards for pollinators can be found in the form of nectar or pollen nutrition, sites for mating and oviposition, shelter from the environment, or particular chemical compounds used for the attraction of conspecifics (Simpson & Neff, 1981). These rewards are advertised with visual signals such as floral colour, patterning, size, or shape of flowers and inflorescences, which are often acting in concert with olfactory signals such as floral volatiles (Willmer, 2011). However, the sheer presence of floral signals does not automatically guarantee insect visitation. Signal and reward are decoupled in flowers, so pollinators have evolved the ability to associate variation in flower signals with the quantity and quality of rewards (Schaefer *et al.*, 2004; Knauer & Schiestl, 2015). Preferences for flower signals can differ

strongly between pollinator species, and are formed on different cognitive levels acting individually or in concert. Most simply, a pollinator species may have innate preferences for certain floral signals and e.g. mainly visit flowers of similar colour (Schiestl & Johnson, 2013). These preferences may further be complemented with receiver bias, *i.e.* other preferences that are not originally connected to flower visitation (Schiestl, 2017). An example of a receiver bias is the general preference of pollinators for exaggerated signals, which may lead to a preferential visitation of larger flowers (Naug & Arathi, 2007). Finally, these preferences may be supplemented with, or even partially overridden by newly learned signal-reward associations, which emerge as a result of previous experience. A classical example is floral constancy, the acquired preference of pollinators for a particular flower type (Schiestl & Johnson, 2013). In consequence, plant-pollinator interactions can promote plant microevolution in two directions. On one hand, changes in pollinator preferences can induce strong pre-pollination selection pressure on floral traits. On the other hand, changes in floral traits can lead to plant reproductive isolation e.g. through induction of a switch of pollinator species (Schiestl & Schlüter, 2009).

Although evolved in the context of mutualistic plant-pollinator interactions, floral signals can also attract plant enemies such as insect or mammal herbivores, which is known as the defence-apparency dilemma (Feeny, 1976). Floral traits are thus often under opposite directional selection imposed by pollinators and herbivores, and may undergo micro-evolutionary changes in response to fluctuations of both pollinator and herbivore assemblages (Gómez *et al.*, 2009; Lucas-Barbosa, 2016). Besides this fine-tuning of floral signals, plants have evolved a wide range of additional response mechanisms to herbivory. Most simply, endurance tactics such as the storage of reserves in roots allow the plant to regrow after a herbivore attack (van der Meijden *et al.*, 1988), but also direct deterrence mechanisms e.g. with spines, trichomes, or toxic secondary metabolites are widespread (Bennett & Wallsgrove, 1994; Hanley *et al.*, 2007). However, some herbivore species have evolved methods to overcome these mechanisms, e.g. by detoxification of plant metabolites (Winde & Wittstock, 2011). Plants in turn have counter-evolved indirect responses and attract herbivore enemies such as parasitoid wasps with leaf volatiles (De Moraes *et al.*, 1998). Changes in herbivore abundance, composition, or behaviour can thus also induce short-term evolution in plant traits not directly related to reproduction, as for example in plant growth rate (Turley *et al.*, 2013). Altogether, a comprehensive understanding of insect-mediated plant microevolution requires knowledge on the connection between mutations of plant traits and changes in insect-induced selection pressures.

Genetic mechanisms behind insect-mediated plant microevolution

Mutations in plant trait pathways

The manifold visual and olfactory signals emitted by a plant can unfold their attractive or repellent effect on insect visitors over a wide range of distances. Nevertheless, the optical resolution of compound insect eyes is low, and most signals such as flower form or pattern act only within a relatively short distance and only after the insect has been initially attracted to the plant by other long-ranging signals (Vorobyev *et al.*, 1997; de Ibarra *et al.*, 2015). The most important of these primary, long-distance signals are flower colour and scent (Balkenius *et al.*, 2006). Up to now, a substantial part of the molecular pathways underlying the biosynthesis of flower colour and, to a lesser degree, flower scent has been characterized (Grotewold, 2006a; Dudareva *et al.*, 2013; Lloyd *et al.*, 2017). Mutations both in structural and regulatory elements of these pathways can change plant attractiveness to insect visitors and thus provide the raw material for intraspecific changes in selection pressure (Coberly & Rausher, 2008; Streisfeld *et al.*, 2013).

Three main types of colour pigments have been described in floral tissue (Grotewold, 2006a). *Carotenoids* are tetraterpenoid compounds with a yellow to orange coloration, which can be divided into two main groups: While carotenes are simple hydrocarbons, xanthophylls additionally contain oxygen (Hirschberg, 2001). Carotenoids accumulate in different types of plastids and, besides contributing to flower colour, are also required for membrane stabilization, light collection, and photoprotection during photosynthesis (Ruiz-Sola & Rodríguez-Concepción, 2012). However, due to their bright and rather limited colour range, carotenoids often function as background coloration and are usually co-occurring with other pigments in darker flowers (Forkmann, 1991). *Betalains* are yellow to red indole derivatives stored in the vacuole. They exclusively occur in the Caryophyllales and thus play no role in other plant groups, including the systems assessed in this work (Hatlestad *et al.*, 2015). *Anthocyanins,* a group of flavonoids, are by far the most important and widespread floral pigments. The structure of the anthocyanin pathway is conserved across the angiosperms and consists of relatively few enzyme-coding and regulatory genes. This has not only facilitated the genetic characterization of anthocyanin production, it also allows the application of a candidate-gene approach to track down the genetic underpinnings of a floral colour change (Wessinger & Rausher, 2012). The source compound of the pathway is the aromatic ketone chalcone, which is biosynthesized by the enzyme chalcone synthase. After initial isomerization to naringenin, the pathway is divided in three branches, which give rise to blue delphinidin, purple pelargonidin, and red cyanidin glycosides. Within these branches, the precursors undergo hydroxylation by branch-specific flavonol/oid hydroxylases, before they are reduced by dihydroflavonol-4-reductase, oxygenated by anthocyanidin synthase, and coupled to sugars by

glycosyltransferases (Tanaka *et al.*, 2008). The final, species-specific hue of flower tissue is usually achieved by further fine-tuning of this pathway scaffold: A series of WD40-bHLH-MYB transcription factor complexes regulates the flux of metabolites through the three pathway branches, resulting in blending of different anthocyanin types (Gonzalez *et al.*, 2008; Li, 2014). In addition, the hue of individual derivatives within the branches is influenced by the pH in the vacuole, co-pigments, metal ions, and further modifications such as the attachment of different sugar groups and chemical residues (Castañeda-Ovando *et al.*, 2009).

Flower colour is an evolutionary labile trait, and comparisons on a macro-evolutionary scale show that pollinator-driven speciation events in various angiosperm families are frequently accompanied by changes in anthocyanin production and composition (Rausher, 2008). Mutations behind these changes can occur in coding or non-coding sequences of both structural and regulatory pathway genes (Wessinger & Rausher, 2012): A change in the coding sequence of a structural pathway gene most likely results in loss of function (LOF) of the corresponding protein. LOF of genes shared by all pathway branches such as *chalcone synthase* or *anthocyanidin synthase* typically blocks the complete anthocyanin production. This usually leads to a transition from coloured to white, yellow, or green flowers depending on co-pigmentation e.g. by carotenoids (Grotewold, 2006b). This transition has been repeatedly observed in many angiosperm lineages and can sometimes be part of a switch in pollination syndrome, e.g. from bee or hummingbird to moth or bat-pollinated flowers (Baker, 1963; Grant, 1994). On the other hand, effects of LOF mutations in genes unique to one branch such as the *flavonoid hydroxylases* usually lead to a redirection of the metabolite fluxes to the other branches, which results in a colour transition. A frequently observed colour transition is the change from blue to red flowers, which is usually caused by a shift from more to less hydroxylated anthocyanins (Wessinger & Rausher, 2012). This can also be associated with a switch in pollination syndrome from bee to hummingbird pollinated flowers (Thomson & Wilson, 2008). The phenotypic effect of the other mutation types, *i.e.* mutations in non-coding sequences of structural genes and mutations in regulatory genes, is context-dependent. Interruption of gene activation can lead to pigment loss, while interruption of gene repression may result in pigment accumulation (Streisfeld & Rausher, 2011). In addition, LOF of elements regulating spatial expression of anthocyanins can change floral patterning (Glover *et al.*, 2013). Investigations in natural populations have shown that loss of pigmentation is generally associated with mutations in regulatory elements and transcription factors, while colour transitions can also be caused by mutations in functional elements. This might be connected to deleterious pleiotropic effects caused by LOF of early pathway genes (Wessinger & Rausher, 2012). Besides flower colouring, anthocyanins play an important role in neutralizing reactive oxygen species formed by overexposure to light and

extreme temperatures (Stapleton, 1992; Qiu *et al.*, 2016), and their complete loss in the whole plant can thus have negative physiological effects. A similar accumulation of mutations in elements with minimal deleterious pleiotropic effects can be observed in the *WD40-bHLH-MYB* transcription factor complexes: The majority of regulatory mutations have been discovered in *MYB* genes, which are much more numerous and specialized in function than the much more conserved *WD40* and *bHLH* genes (Quattrocchio *et al.*, 1993; Streisfeld & Rausher, 2011).

In contrast to flower colour, biosynthesis of floral scent is much more diverse, and the main commonality of floral volatile organic compounds (VOCs) is their ability to be actively or passively transported to, or synthesized at the cell surface to be released in the ambient environment (Maffei, 2010). Most VOCs are thus lipophilic secondary metabolites with a high vapour pressure and low molecular weight (Dudareva *et al.*, 2013), but there are exceptions such as the long-chained alkenes produced by some *Ophrys* orchids (Schiestl *et al.*, 2000). Although more than 1700 different plant VOCs have been described up to now (Knudsen *et al.*, 2006), production of the great majority of them is based on only a handful of primary metabolites. VOCs are thus commonly attributed to one of four major compound classes (Dudareva *et al.*, 2013). However, there are also other classification systems in use (Knudsen *et al.*, 2006; Schiestl, 2010), and many VOCs have properties from multiple classes. *Aromatics* are compounds containing benzene carbon rings without other elements such as nitrogen or sulphur. They are synthesized via the phenylpropanoid pathway with phenylalanine as source metabolite. *Terpenoids* are a highly diverse group of hydrocarbon VOCs and are produced by condensation of multiple units of two isoprenes, isopentenyl diphosphate, and dimethylallyl diphosphate. *Fatty acid derivatives* are carboxylic acids coupled to long, saturated and unsaturated aliphatic chains. Many fatty acid derivatives are produced from linoleic or linolenic acid through the lipoxygenase pathway. *Amino acid derivatives* are a heterogeneous group of VOCs deriving from amino acids such as serine, valine, leucine, alanine, isoleucine, and methionine. Through various enzymatic processes (de-, transamination, decarboxylation, oxidation etc.), the amino acids are turned into aldehyde intermediates and further chemically modified to VOCs. Amino acid derivatives usually contain nitrogen or sulphur groups (Dudareva *et al.*, 2013). As in floral colour, the final blending of VOCs into often complex floral bouquets allows a fine-tuning of the floral signal (Dicke *et al.*, 2009; Kessler *et al.*, 2013).

Scent bouquet compositions can vary greatly even within populations (Knudsen, 2002), and can evolve quickly in response to both insect-mediated and artificial selection pressures (Zu *et al.*, 2016; Gervasi & Schiestl, 2017). However, measurements with electro-antennographic detectors (EAD) have shown that often only a fraction of VOCs within a floral bouquet elicits responses in sensory neurons of insects. This set of physiologically active VOCs can be very

different between insect species, complicating predictions on the micro-evolutionary relevance of changes in floral scent (Schiestl, 2015). The best understood cases of VOC mutations and their consequences thus stem from highly specialized plant-insect systems, which have evolved private channels of communication by using an often very limited set of unusual compounds (Raguso, 2008). The bouquet of these plants often mimics scent emitted by other sources, as e.g. in carrion flowers which attract fly pollinators with an odour resembling rotten meat (Jürgens *et al.*, 2013; Jürgens & Shuttleworth, 2015), or in sexually deceptive orchids, which are pollinated by pseudocopulation of male insects mistaking the flower for a female partner (Peakall & Beattie, 1996; Schiestl *et al.*, 1999). Some species within the sexually deceptive orchid genus *Ophrys,* for example mimic the cuticular sex pheromones of solitary bees, consisting of a blend of alkenes with different length and double bond positions (Schlüter *et al.*, 2011). Investigations of two closely related *Ophrys* species suggest that a *stearoyl-acyl-carrier-protein desaturase* (*SAD*) responsible for double bonds introduction at position 7 may have undergone subcellular relocalization after initial duplication, enabling it to introduce double bonds at positions 7 and 9. This imperfection in pheromone mimicry could have lead to pollinator-mediated deleterious pleiotropy, out of which the species may have escaped through a switch to a new pollinator species by dropping the production of the ancestral 9-alkenes (Sedeek *et al.*, 2016). In summary, mutations in plant traits, although emerging at random, are more likely to contribute to insect-mediated plant microevolution if they occur in pathway elements with minimal pleiotropic and optimal ecological effects.

Insect-mediated selection of plant traits

After new gene variants have arisen through mutation, the different alleles undergo changes in frequency. Due to genetic drift, gene flow, and linkage of mutations to other pleiotropic traits under selection, allele frequencies change even if the mutation is neutral (Kimura, 1983; Birky & Walsh, 1988; Ellstrand, 2014). Since genetic drift is based on random sampling of alleles between parent and offspring, its efficacy is negatively correlated with population size, and it can even overpower natural selection if the allele frequency is small enough (Masel, 2011). However, changes in plant traits involved in biotic interactions are very likely to have a fitness effect, and the regularity of some macro-evolutionary patterns such as pollination syndromes suggests that in general, selection plays a more important role than genetic drift in the evolution of these traits (Wessinger & Rausher, 2012). *Insect-mediated stabilizing selection* can be seen as the null model of insect-mediated plant-microevolution. In this case, the ancestral, fixed allele is at fitness maximum, and any mutation causing a phenotypic deviation in either direction (*e.g.* brighter or darker flower colour) will get lost over time. This leads to a loss of genetic diversity and a decrease of trait variance, resulting in morphological stasis of this trait (Campbell, 2009). The prevalence of

stabilizing selection in nature is still debated, and while the fossil-based theory of punctuated equilibrium suggests that stasis is the default state for the majority of traits (Charlesworth *et al.*, 1982; Estes & Arnold, 2007), stabilizing selection is hardly detected in extant systems (Kingsolver *et al.*, 2001). However, the strength of stabilizing selection may simply fall below the detection limit once a population has adapted to a fitness peak (Haller & Hendry, 2014).

Several alternative models of insect-mediated selection can lead to micro-evolutionary change in plant traits. *Insect-mediated directional selection* describes the case where an allele conferring an extreme plant phenotype (*e.g.* white flower colour) has the highest relative fitness (Campbell, 2009). It is often associated either with migration of a plant species to new habitats, or with a change in the composition or preference of insect visitors in an established population (Latta, 2010). Directional selection usually leads to a rapid phenotypic transition and local adaptation of the population as a whole, but does not preserve genetic variation in the end. However, if a species is divided in multiple populations, the composition and behaviour of the insect community can be different between them (*e.g.* selection for white flower colour in one, and red colour in another population), leading to inter-population differences in the direction and strength of trait selection (Waser & Campbell, 2004). In this case, which is known as *insect-mediated divergent selection,* reproductive barriers can emerge between populations, ultimately leading to macroevolution through so-called ecological speciation (Schluter, 2009). Divergent selection can also occur within a single population: In *insect-mediated disruptive selection*, the fitness of both extreme phenotypes (*e.g.* black and white flower colour) is superior over intermediates (Campbell, 2009). Disruptive selection is assumed to occur either when a plant population is adapted to two or more pollinator-, or herbivore species differing markedly in a selective trait (*e.g.* body size), or if both the plant and the pollinator population show a continuum in the dimension of reciprocally adapted traits (*e.g.* corolla tube length and proboscis length). In the latter case, selection would occur because plant individuals at the edge of the trait distribution experience reduced competition for pollinators (Waser & Campbell, 2004). Disruptive selection can lead to a macro-evolutionary lineage split via ecological speciation, although this is only one of several possible outcomes (Rueffler *et al.*, 2006). However, the strength of divergent and disruptive selection can vary greatly over time, and cases in which the speciation process remained incomplete are not uncommon (Nosil *et al.*, 2009). In the cases discussed here, ecological speciation is more likely to complete if divergent or disruptive selection affects traits conferring insect-mediated reproductive isolation between lineages. This so-called floral isolation can be induced mechanically through mutation of traits important for the specificity of pollen transfer (e.g. the position of anthers and stigma), and/or ethologically through mutation of traits inducing a change in pollinator behaviour such as floral constancy (Grant, 1994; Schiestl & Schlüter, 2009). A special case among micro-

evolutionary models is *insect-mediated balancing selection.* Unlike in the previously explained selection types, where the preservation of intraspecific allelic variation is transient, balancing selection never leads to macroevolution, and all alleles co-exist (theoretically) indefinitely as protected polymorphisms (Fijarczyk & Babik, 2015). Balancing selection can even persist if lineages undergo speciation through other selective forces later on, leading to trans-specific polymorphisms (Wiuf *et al.*, 2004). According to Dobzhansky, *"The absolute equality of two biological forms is* [...] *highly unlikely"* (Dobzhansky, 1951). Nevertheless, many insect-relevant plant traits are polymorphic (Weiss, 1995; Galen, 1999), hence the mechanisms of balancing selection need further explanation.

A balancing selection regime can arise if a trait is under directional, divergent or disruptive selection, but opposing mechanisms prevent the fixation of either allele and the macro-evolutionary transition of the species (Gray & McKinnon, 2007). In *antagonistic pleiotropy,* insect-mediated directional selection on a trait counteracts selective pressures on other pleiotropic traits of the same locus, which results in a net stalling of allele frequencies (Williams, 1957). This has been documented for example in *Protea* species with floral colour polymorphism, where one particular morph has a pleiotropic advantage in reproduction, but experiences a disadvantage in survival as it is preferentially attacked by herbivores (Carlson & Holsinger, 2010). Under insect-mediated divergent or disruptive selection, a protected polymorphism can arise through *spatiotemporal heterogeneity* of the insect community. Insect communities can thereby be heterogeneous both between entire plant populations and between microsites or environmental gradients within a single population. In spatially varying selection, divergent or disruptive selection is simply counteracted by constant gene flow between the sites (Delph & Kelly, 2014). Mathematical models have shown that such conditions can be met, although only under a clearly defined parameter space with ample differences between sites and spatially limited dispersal (Levene, 1953; Christiansen, 1974). In temporally varying selection, the composition of the insect community changes periodically within a site, preferring either one or the other extreme phenotype. Unlike in disruptive selection, the two alleles are thus not simultaneously, but rather alternating on a fitness maximum, preventing fixation of either of them (Delph & Kelly, 2014). Again, such conditions can only arise in a narrow parameter space (Hedrick, 1976), but there are known examples of plant-herbivore dynamics, where e.g. a successional change in the local herbivore community temporarily switches the defence strategy from resistance to tolerance (Hakes & Cronin, 2011). However, temporally varying selection does not necessarily require a change of the insect community. In *negative frequency-dependent selection* (NFDS), morph frequencies oscillate because the selective advantage of each morph is negatively correlated with its abundance (Fisher, 1930; Clarke & O'Donald, 1964). Insect-mediated NFDS is often mentioned in the context of

rewardless plants, most prominently in the orchid *Dactylorhiza sambucina,* where it has been proposed that pollinators may learn to avoid common morphs more quickly than rare morphs (Gigord *et al.*, 2001). Another pollinator-linked case of NFDS can be found in heterostylous plants, where anthers and stigma are reciprocally positioned within the flowers of different morphs, promoting outcrossing between them (Eckert *et al.*, 1996; Barrett & Shore, 2008). Empirical demonstration of the action of insect-mediated NFDS has been challenging so far (Imbert *et al.*, 2014), and in the case of *D. sambucina,* the polymorphism is likely maintained by other factors such as differences in seed viability (Pellegrino *et al.*, 2005; Jersáková *et al.*, 2006).

Under particular circumstances, a balanced selection regime can also arise under insect-mediated stabilizing selection, the null model of insect-mediated plant microevolution. If two alleles show (incomplete) co-dominance, the intermediate phenotype of heterozygous individuals can be at the fitness maximum, *e.g.* because a new adaptive mutation can overshoot the phenotypic optimum in homozygous state (Manna *et al.*, 2011; Sellis *et al.*, 2011). Individuals homozygous for both alleles will then persist in the population due to Mendelian segregation, and neither of the alleles will be fixed in the population. This case, where a single-locus heterozygote in a single population within a single environment has a higher fitness than either homozygote, is known as *overdominance* (Charlesworth & Willis, 2009; Delph & Kelly, 2014). The term overdominance is inconsistently connoted in the literature. Often, it is used synonym to *heterozygote advantage*, which, however, can also occur under some of the previously described types of balancing selection (Hedrick, 2012). In the context of heterosis, overdominance is not necessarily associated with fitness, and just describes a condition where the phenotype of the heterozygote lies outside of either homozygote parent (Chen, 2013). Initially used as an explanation for inbreeding depression and hybrid vigour in crop plants, overdominance was later adopted to population genetics by Dobzhansky (Dobzhansky, 1951). Unlike the classic hypothesis, which stated that most mutations are deleterious and wild-type alleles should be fixed at most loci, the balance hypothesis of Dobzhansky argued that almost all polymorphisms in natural populations are maintained by overdominance (Dobzhansky, 1955). Dobzhansky's hypothesis was widely discussed, splitting the scientific community in a "classical" and "balance school" (Mayr, 1982). However, over time, the interest in overdominance has diminished. Partially because the focus has moved to the other, previously mentioned types of balancing selection, but mainly because it turned out to be surprisingly difficult to verify the action of overdominance in natural systems (Gray & McKinnon, 2007; Delph & Kelly, 2014; Draghi & Whitlock, 2015). The main issue is that several other, more complex genetic situations can cause a net effect analogous to overdominance. Inbreeding depression for example is often associated with increased homozygosity, but this is due to *dominance, i.e.* the exposure of deleterious

recessive alleles (Charlesworth & Charlesworth, 1999). If two such deleterious recessive alleles were reciprocally homozygous in each parent (Ab/Ab and aB/aB), the heterozygous offspring (Ab/aB) would have a higher fitness than each parent. In case only one of the alleles was known, this *associative overdominance* would resemble true overdominance (Ohta & Kimura, 1970; Charlesworth & Willis, 2009). Another such situation occurs under *emergent overdominance*, where heterozygote advantage results as a net fitness effect after averaging the fitness at a particular locus over multiple spatial or temporal units of a species, *e.g.* populations or generations (Delph & Kelly, 2014). A clear-cut demonstration of overdominance thus requires knowledge on the genes and alleles under selection, on the relative fitness of each genotype, and on the mechanism of selection (Hedrick, 2012). Up to now, only a handful of putative cases of insect-mediated overdominance in plant traits have been described (Mojonnier & Rausher, 1997; Malerba & Nattero, 2012; Takahashi *et al.*, 2015), but none of these cases is completely understood, let alone unequivocally demonstrated to be overdominant.

Epigenetic mechanisms behind insect-mediated plant microevolution

As outlined in the previous section, variation of plant traits is created and transmitted on the genetic level, while insect-mediated selection among this variation operates on the phenotypic level. However, the connection between genotype and phenotype, *i.e.* plant trait development, has not been discussed in-depth so far. In the early 1940s, Conrad Waddington was among the first biologists who realized that development and genetics are related (Deans & Maggert, 2015). Waddington defined "*the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being*" as epigenetics (Waddington, 1942). In the following decades, experimental evidence accumulated that epigenetics also has a heritable component: In order for different organs to be formed and maintained by cellular differentiation, a single genome does not only give rise to multiple epigenomes, the settings of these individual epigenomes also have to be mitotically transmitted from one cell generation to the next (Hadorn, 1974). The proposition of mechanisms in which epigenetic information is superimposed on DNA finally lead to the assumption that epigenetic variation can also be meiotically transmitted between generations (Holliday, 2006). This required a redefinition of epigenetics, *e.g.* as "*The study of mitotically and/or meiotically heritable changes in gene expression that occur without a change in DNA sequence*" (Riggs *et al.*, 1996). Since then, several examples of heritable epigenetic changes have been characterized, most of them in plants. Some of these changes have dramatic consequences on the phenotype, *e.g.* a change from bilateral to radial floral symmetry (Cubas *et al.*, 1999). Epigenetic mutations

(*epimutations*) can thus potentially be subject to the same insect-mediated selection pressures as genetic mutations, and could play a significant role in plant microevolution.

A simple way of meiotic transmission of information is the storage of gene products in the cytoplasm of egg cells. Such a *parental effect* can induce trans-generational phenotypic plasticity in the descendants, priming them for an environmental change such as the arrival of a new herbivore (Agrawal, 2002). However, cytoplasmic parental effects only last until the next plant generation, and a transmission of epigenetic changes over more than one generation requires a meiotically transmissible epigenetic encoding system similar to DNA sequence (Grossniklaus *et al.*, 2013). Perhaps the best-known epigenetic encoding is *DNA methylation*, the addition of methyl groups to the 5th position of the pyrimidine ring of cytosine, or less commonly, adenine (Ratel *et al.*, 2006). In *Arabidopsis,* around 32.4% of all cytosines are methylated, 24% of which occur in CG sequence context, 6.7% in CHG context, and 1.7% in CHH context (H stands for A, C, or T). In most regions of the genome, CG, CHG, and CHH methylation are highly correlated and show enrichment in repeat-rich pericentromeric regions (Cokus *et al.*, 2008). In those regions, DNA methylation mainly prevents the expression of transposable elements, which is probably its ancestral function in all eukaryotes (Yoder *et al.*, 1997). Also, methylation of promoter sites usually leads to silencing of the corresponding gene (Zemach *et al.*, 2010). However, within gene bodies, methylation occurs almost exclusively in CG context (Cokus *et al.*, 2008), where it may be associated with splicing regulation and silencing of internal promoters and transposons (Maunakea *et al.*, 2010; Maor *et al.*, 2015). DNA methylation is thus essential for plant development and leads to abnormal phenotypes if perturbed (Finnegan *et al.*, 1996). De-/methylation of cytosines is mediated by DNA methyltransferases (DMTs), which fall into two main classes: *Maintenance* DMTs copy methylation patters from the parental to the daughter strands during DNA replication, and *de-novo* DMTs add or remove methyl groups from particular sites on the genome (Finnegan & Kovac, 2000). Unlike in mammals, where DNA methylation patterns are completely reset during gametogenesis and early embryonic development, reprogramming in plants is somewhat relaxed, allowing for the transmission of such *de-novo* DNA methylation changes (Kawashima & Berger, 2014). In the context of plant-insect interactions, the transmission of *de-novo* DNA methylation changes is supported by three lines of evidence, which, however, have hardly been studied simultaneously in the same system: (1) Some insect-induced phenotypic changes can be transmitted to offspring, sometimes even over multiple generations. Herbivory can for example induce trans-generational changes in levels of defensive leaf compounds (Ballhorn *et al.*, 2016) or in trichome density (Scoville *et al.*, 2011). (2) Interactions with herbivore insects can induce genomic de-/methylation in plants, which can be transmitted to offspring (Verhoeven *et al.*, 2010; Rasmann *et al.*, 2012). (3) DNA methylation changes can sometimes induce

dramatic phenotypic alterations in traits relevant to insect visitors. For example, the before mentioned change in *Antirrhinum* floral symmetry is due to methylation of the *Lcyc*-gene (Cubas *et al.*, 1999), and the development of herbivore-deterring spines in *Ilex aquifolium* is correlated with DNA methylation changes (Herrera & Bazaga, 2013).

Another type of epigenetic encoding is associated with the compaction of DNA to chromatin: Similar to a spool, 146 base pairs of DNA are wound around a nucleosome, consisting of four dimeric core histones, and are securely locked by a single linker histone in condensed state (Cutter & Hayes, 2015). Histone proteins can be subject to post-translational chemical *histone modification*, especially on their long unstructured *N*-terminal tails. Modifications include methylation, acetylation, phosphorylation, ubiquitination, and many more (Bannister & Kouzarides, 2011). The sequence of modified amino acids along a histone tail thereby forms a "histone code", which, besides initiating condensation of chromosomes and marking damaged DNA sections for repair, also controls gene transcription (Jenuwein & Allis, 2001). In addition, transcription can also be influenced by the exchange of individual histones with other histone variants (Talbert & Henikoff, 2010). Chromatin remodelling in plants has been observed upon various abiotic stresses (Luo *et al.*, 2012) as well as bacterial infections (Jaskiewicz *et al.*, 2011), but similar studies on plant-insect interactions are lacking so far. However, it is likely that chromatin remodelling plays an important role in herbivore deterrence: Histones can be modified by the plant defence hormones salicylic and jasmonic acid (Caarls *et al.*, 2015), and there seems to be extensive crosstalk between some histone modifications and DNA methylation (Du *et al.*, 2015).

The third epigenetic encoding, *RNA interference* (RNAi), is well conserved across eukaryotes and plays an essential role in post-transcriptional silencing of viral, transposal, or endogenous genes. It is triggered by the presence of two species of double-stranded RNA (dsRNA): short-hairpin forming pri-microRNA or longer dsRNA fragments, *e.g.* from a retrovirus. Dicer ribonuclease proteins cleave these RNA-types into pieces of 20-25 bp length. If the template was a pri-microRNA, these pieces are known as microRNA (miRNA), whereas cleaved fragments from long dsRNA are known as small-interfering RNA (siRNA) respectively. These pieces are incorporated into the RNA-induced silencing (RISC) Argonaute protein complex, which then degrades nascent mRNA complementary to the incorporated RNA piece and prevents translation (Fire *et al.*, 1998; Castel & Martienssen, 2013). RNAi plays an important role in many plant-insect interactions, particularly in the deterrence of herbivores (Huang *et al.*, 2016). However, experiments in *A. thaliana* have shown that RNAi can also lead to RNA-dependent *de-novo* DNA methylation (RdDM): The DNA regions to be methylated are transcribed, turned into dsRNA, cleaved into 24 bp long siRNA, and loaded into the RdDM complex. This complex contains a catalytically active DNA methyltransferase, which methylates DNA cytosines in any sequence

context at sites complementary to the loaded siRNA (Wassenegger *et al.*, 1994; Zhang & Zhu, 2011). RdDM is an important factor in many processes such as transposon silencing, stress response, reproduction, and interallelic as well as intercellular communication (Matzke & Mosher, 2014), and controls the expression of evolutionary relevant plant traits such as the self-incompatibility system in *Brassica* (Tarutani *et al.*, 2010). It is therefore likely that RdDM is a key mechanism in plant-insect interactions and induces heritable DNA-methylation changes as described above.

In summary, there is some evidence that epigenetic encoding has essential similarities to genetic encoding, including the occurrence of heritable mutations affecting phenotypic expression (Henderson & Jacobsen, 2007; Hauser *et al.*, 2011). However, average rates of epimutations are not only much higher than observed from DNA (van der Graaf *et al.*, 2015). As mentioned before, they can also be induced or even reverted by the cellular machinery upon external triggers, e.g. from an insect visitor (Gutzat & Scheid, 2012). This important difference has initiated a discussion on whether epigenetic encodings could undergo *Lamarckian evolution,* an evolutionary concept which was outlined by Jean-Baptiste Lamarck in 1809 and thus predates Darwin's and Wallace's theory on the origin of species (Lamarck, 1809; Jablonka & Lamb, 2008; Holeski *et al.*, 2012). Unlike in Darwinian evolution, which assumes that the occurrence of mutations is a random process, Lamarckian evolution proposes that an organism can pass on acquired characteristics to offspring, implying that the environment influences both selection *and* mutation. The reconciliation of Darwinian evolution with Mendelian inheritance has rendered Lamarck's evolutionary theory obsolete, and it was eventually abandoned in the course of the modern synthesis. Apart from the notion that the reference to Lamarck's work may be historically inappropriate (Burkhardt, 1995), there is still no evidence that epigenetic inheritance does not fit in a Darwinian evolutionary concept: Epigenetic encoding is always coupled to DNA sequence, so genetic and epigenetic evolution are not exclusive or alternative processes (Penny, 2015). While the environment can indeed trigger changes in epigenetic encoding, there is no evidence so far that these changes can be exclusively site-specific (Roquis *et al.*, 2016). Of these epimutations, only a fraction, if any, affect the germline, and it is implausible that only the adaptive acquired epimutations are not reset during embryogenesis (Latta, 2010). Even if adaptive, environmentally induced epigenetic mutations are heritable, it remains questionable whether the high epimutation rate would allow them to persist over an evolutionary relevant time (Heard & Martienssen, 2014; Verhoeven *et al.*, 2016). However, regardless of the mode of inheritance, the question remains to which extent epigenetic changes contribute to insect-mediated plant microevolution.

Studying insect-mediated plant microevolution

Traditionally, insect-mediated plant microevolution has been studied in the context of population genetics and evolutionary ecology. Population genetics approaches include the study of genetic variation and changes in allele frequency due to the genetic and epigenetic mechanisms explained above. On the other hand, evolutionary ecology approaches focus on the interactions between plants and insect visitors that underlie the observed micro-evolutionary changes (Fernández-Mazuecos & Glover, 2017). These approaches are still standard and timely especially for the analysis of the still poorly known epigenetic aspects of microevolution. However, concerning genetic mechanisms of microevolution, accumulation of knowledge gained from model systems as well as technological advance now allows to shift the focus from the lab to the field. It is now possible to unify those previously separate fields of research and study the connection between ecological, evolutionary, and developmental processes in natural populations of non-model systems (Abouheif *et al.*, 2014). Such an eco-evo-devo approach allows a complete reconstruction of the trajectories of insect-mediated micro-evolutionary processes, starting from mutation over the generation of intraspecific polymorphisms to the selection pressures imposed by insect visitors and the onset of reproductive isolation.

In general, micro-evolutionary processes take much less time than large macro-evolutionary transitions. Under laboratory or greenhouse conditions, it is thus even possible to monitor complete micro-evolutionary processes in real time, provided that a sufficiently strong (artificial) selection pressure is applied to a model organism with sufficiently short generation time. However, in natural populations, time constraints usually still impede a complete tracking and recording of micro-evolutionary events from the occurrence of a new mutation to the final evolutionary outcome (Merilä *et al.*, 2001). Therefore, naturally occurring micro-evolutionary events are commonly studied retrospectively using populations with already established polymorphisms. In case the polymorphism has a genetic basis, reconstruction of the micro-evolutionary events typically includes the localization of the underlying mutation(s) as well as the characterization of the selective forces acting on them. On the other hand, polymorphisms with an epigenetic basis would be much more difficult to assess, since it requires a prior exclusion of any confounding genetic or parental effects. In the case of insect-mediated plant microevolution, a considerable number of polymorphic plant systems have been at least partially characterized so far; the most notable ones are listed in Table 1.

Table 1 | Plant species with notable insect-(co)induced polymorphisms in floral/leaf colour, scent, shape, or a combination hereof. For some of them, the underlying mutated gene(s) and/or the selective force has been characterized as well.

Polymorphism	Mutated gene	Selective force	Reference
Floral colour			
White / Pink / Purple *Cirsium palustre*	Unknown	Antagonistic pleiotropy Bees & Abiotic factors	(Mogford, 1974b) (Mogford, 1974a)
White / Pink *Protea* spp.	Unknown	Antagonistic pleiotropy Bees & Physiological factors	(Carlson & Holsinger, 2010)
Blue / Red *Lysimachia arvensis*	Unknown Single locus	Antagonistic pleiotropy Bees & Abiotic factors	Ortiz *et al.* (2015)
White / Yellow / Pink / Bronze *Raphanus sativus*	Unknown Two loci	Antagonistic pleiotropy Pollinators & Herbivores	Irwin *et al.* (2003)
White / light / dark Purple *Ipomoea purpurea*	*CHS* Transposon	Antagonistic pleiotropy Bees & Physiological factors	Coberly and Rausher (2008)
Yellow / Purple *Dactylorhiza sambucina*	Unknown	Neg. frequency-dependence (?) Bees	Groiß *et al.* (2017)
White / Red / Black *Gymnadenia rhellicani*	*R2R3-MYB* SNP / Deletion	Overdominance Bees & Flies	Kellenberger *et al.* (Chapter III)
White / Purple *Sisyrinchium* sp.	Unknown Single locus	Overdominance (?) Bees & Sp. interference	Takahashi *et al.* (2016)
Blue / Purple *Phlox drummondii*	*R2R3-MYB*	Divergence (Reinforcement) Butterflies	(Hopkins & Rausher, 2011)
Orange / Red *Disa ferruginea*	Unknown	Divergence Butterflies	Newman *et al.* (2012)
White / Purple *Anacamptis coriophora*	Unknown	Unclear Bees	(Dormont *et al.*, 2014)
Floral colour and scent			
Yellow / Red Scent bouquet correlation *Mimulus aurantiacus*	*R2R3-MYB* (+minor genes)	Divergence Bees & Hawkmoths	Streisfeld *et al.* (2013) (Büsser, 2004)
Yellow / Orange / Red Linalool *Dactylorhiza romana*	Unknown	Unclear Bees	(Salzmann & Schiestl, 2007)
White / Lilac / Purple Terpenoid DMNT *Calanthe sylvatica*	Unknown	Unclear Hawkmoths	(Delle-Vedove *et al.*, 2011)
Yellow / Purple Terpenoids *Iris lutescens*	Unknown	Unclear Bees	(Imbert *et al.*, 2014) (Wang *et al.*, 2013)
White / Purple Shikimic pathway VOCs *Orchis mascula*	Unknown	Unclear Bees	(Dormont *et al.*, 2014) (Dormont *et al.*,

			2010)
White / Purple Shikimic pathway VOCs *Orchis simia*	Unknown	Unclear Bees	(Dormont *et al.*, 2014)
Floral colour and shape			
White / Pink / Purple Corolla size & Flower number *Cosmos bipinnatus*	Unknown Single locus	Overdominance (?) Bees	Malerba and Nattero (2012)
16 Orange morphotypes Number & texture of spots *Gorteria diffusa*	*R2R3-MYB* (?) (+ unknown papillae genes)	Unclear Flies	(Ellis *et al.*, 2014) (Thomas *et al.*, 2009)
Floral scent and shape			
Sweet / Skunky Corolla size *Polemonium viscosum*	Unknown	Disruptive Bees / Flies	(Galen *et al.*, 1987) (Galen, 1985)
Floral shape			
Actinomorphy / Zygomorphy *Erysium mediohispanicum*	Unknown	Directional Beetles / Flies / Butterflies	(Gómez *et al.*, 2006)
Heterostyly (Distyly) *Primula spp.*	*S*-Locus supergene	Neg. frequency-dependence Bees	(Li *et al.*, 2016) (Keller *et al.*, 2014)
Heterostyly (Distyly) *Narcissus tanzetta*	Unknown	Neg. frequency-dependence Bees, Hoverflies, Moths	(Arroyo & Dafni, 1995)
Heterostyly (Distyly) *Narcissus albimarginatus*	Unknown Polygenic	Unclear	(Pérez *et al.*, 2004)
Heterostyly (Tristyly) *Narcissus pallidulus*	Unknown Polygenic	Unclear Bees	(Pérez *et al.*, 2004)
Leaf shape			
Trichome density *Arabidopsis lyrata*	*GLABROUS1*	Divergent Insect herbivores	(Kivimäki *et al.*, 2007)

Aim and outline of this thesis

The present work aims at contributing to our understanding of the developmental pathways, ecological processes, and evolutionary consequences underlying insect-mediated plant microevolution. To this end, two important yet unresolved micro-evolutionary hypotheses were tested with a broad range of techniques: In chapter I and II, the novel hypothesis that epigenetic alterations contribute to microevolution of plant-insect interactions was tested in a plant-herbivore model system under laboratory conditions. In chapter III, a novel eco-evo-devo approach was applied to a natural, polymorphic population of a non-model plant system to find evidence for Dobzhansky's classic hypothesis that polymorphisms can arise through insect-mediated overdominance.

Chapter I uses a bi-directional approach to study the connection between DNA methylation changes, plant phenotypic changes, and changes in insect behaviour. In a first step, plants of an inbred *Brassica rapa* line were challenged with herbivory of *Pieris brassicae* caterpillars, as well as with the application of the plant stress hormone jasmonic acid. Subsequently, the impact of these stress treatments on the plant methylome was quantified with methylation-sensitive amplified fragment length polymorphisms (MSAP). In addition, resulting changes in plant phenotype and volatile bouquet composition were documented with morphological measurements and gas chromatography - mass spectrometry detection (GC-MSD). In a second step, DNA methylation patterns of a rapid cycling *B. rapa* line were stochastically perturbed with the DNA-methyltransferase inhibitor 5-azacytidine, and the resulting phenotypic changes were recorded as described above. In addition, the effect of these epigenetically induced phenotypic changes on pollinator perception was documented in dual-choice assays with the pollinator *Bombus terrestris.*

Chapter II uses a multi-generation approach to study the retention, transmission, and impact of herbivory-induced phenotypic changes. Two subsequent generations of a rapid-cycling *B. rapa* plant line were subjected to caterpillar herbivory by the specialist *P. brassicae* and the generalist *Mamestra brassicae*. The experimental setup controlled for both genetic and epigenetic variation by using 120 descendant plant lines of a single, self-compatible, and highly inbred *B. rapa* plant. Herbivory-induced morphological changes as well as changes in seed production, leaf-, and flower volatile emission were recorded in both plant generations with morphological measurements and GC-MSD. In addition, the impact of these changes on interactions with parasitoid and pollinator insects was experimentally determined: Herbivore-induced changes in the attraction of the wasp *Cotesia glomerata,* a parasitoid of *P. brassicae,* were assessed in olfactometer assays, and changes in the attraction of the pollinator *Bombus terrestris* were assessed in dual-choice assays. After two consecutive generations of herbivory, the retention of the recorded herbivory-induced

changes as well as their effect on parasitoids and pollinators was assessed for two additional plant generations without herbivory.

Chapter III uses an eco-evo-devo approach to study the emergence of a floral colour polymorphism in the Alpine orchid *Gymnadenia rhellicani. G. rhellicani* is a rewarding (nectar-producing) plant, whose chocolate-like floral odour attracts a broad range of insect pollinators, including bees, flies, butterflies, and moths. Its compact inflorescence usually bears around 30-40 flowers with dark-red to almost black coloured petals. However, in one *G. rhellicani* population on Seiser Alm, South Tyrol, Italy, floral colour is polymorphic with ca. 28% bright red and ca. 10% white coloured individuals. Data on morph frequencies from the last twenty years suggest that the polymorphism in this population is maintained by balancing selection, most probably overdominance. An array of investigation methods was used to assess the developmental molecular basis, the pollinator ecology, and the evolutionary background of this previously unstudied floral colour polymorphism: (1) To assess the molecular *development* of the polymorphism, anthocyanin and carotenoid colour pigments were first quantified in flower tissue of all three morphs using high-pressure liquid chromatography coupled with tandem mass-spectrometry (HPLC-MSMS). Second, pigment levels were correlated with differences in gene expression, which were determined with a high-multiplexing RNA-seq approach, yielding differentially expressed candidate genes involved in pigment production. Third, states of nucleotide polymorphisms were correlated with gene expression levels in a transcriptome-wide association study (TWAS) to find causative genetic mutations of this polymorphism. (2) To assess the impact of this polymorphism on the *ecology* of plant-pollinator interactions, pollinator-relevant phenotypic plant traits were first compared among the three colour morphs. The measured traits include plant height, flower number, flower shape, inflorescence temperature, as well as differences in the composition and emission rates of flower volatiles. Second, the reproductive system and pollinator dependence of *G. rhellicani* was assessed with ploidy measurements and pollinator-exclusion experiments (caged plants). Third, the preferences of the different pollinator taxa were assessed in choice assays by placing time-lapse cameras in front of the plant morphs in the population. (3) To understand the *evolution* of the polymorphism, the state of the mutated gene determined under (1) was determined in 13 wild-type *G. rhellicani* populations across its Alpine habitat, as well as in a second polymorphic population on Monte Bondone, Trento, Italy.

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Chapter I Herbivore-induced DNA demethylation changes floral signalling and attractiveness to pollinators in *Brassica rapa*

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Abstract

Plants have to fine-tune their signals to optimise the trade-off between herbivore deterrence and pollinator attraction. An important mechanism in mediating plant-insect interactions is the regulation of gene expression via DNA methylation. However, the effect of herbivore-induced DNA methylation changes on pollinator-relevant plant signalling has not been systematically investigated. Here, we assessed the impact of foliar herbivory on DNA methylation and floral traits in the model crop plant *Brassica rapa*. Methylation-sensitive amplified fragment length polymorphism (MSAP) analysis showed that leaf damage by the caterpillar *Pieris brassicae* was associated with genome-wide methylation changes in both leaves and flowers of *B. rapa* as well as a downturn in flower number, morphology and scent. A comparison to plants with jasmonic acid-induced defence showed similar demethylation patterns in leaves, but both the floral methylome and phenotype differed significantly from *P. brassicae* infested plants. Standardised genome-wide demethylation with 5-azacytidine in five different *B. rapa* full-sib groups further resulted in a genotype-specific downturn of floral morphology and scent, which significantly reduced the attractiveness of the plants to the pollinator bee *Bombus terrestris.* These results suggest that DNA methylation plays an important role in adjusting plant signalling in response to changing insect communities.

Introduction

In order to maximise their fitness, organisms have to invest both in their survival and in reproduction. In the case of insect-pollinated plants, there is a strong trade-off in resource investment as the two processes are often competing - not only on a metabolic, but also on an ecological level (Coley *et al.*, 1985; Herms & Mattson, 1992; Byers & Schiestl, 2015): plant defence measures can deter pollinators (Kessler & Halitschke, 2009; Barber *et al.*, 2012), and signals attractive to pollinators can also attract herbivores (McCall & Irwin, 2006; Theis & Adler, 2011), a phenomenon known as the defence-apparency dilemma (Feeny, 1976). Since herbivore and pollinator compositions can fluctuate in time and space, plants need a system to quickly react to alterations of the surrounding insect community (Agrawal, 2001). A candidate mechanism mediating this response is the (de-) methylation of DNA cytidine residues, which can influence gene transcription in a sequence context-dependent manner (Zilberman *et al.*, 2007; Jones, 2012). In recent years, DNA methylation has gained a lot of attention, since changes in the methylome have been shown to be partially heritable in plants (Calarco *et al.*, 2012; Niederhuth & Schmitz, 2014). However, our understanding of the influence and relevance of DNA methylation on traits shaping defence-reproduction trade-offs is still fragmentary.

The diploid model crop plant *Brassica rapa* L. is an ideal system for studying plant-insect relationships as it is visited by a broad range of pollinator (Rader *et al.*, 2009) and herbivore species (Lamb, 1989). Mediated mainly by jasmonate plant hormones, herbivory induces both direct (e.g. via leaf glucosinolates) and indirect defence reactions in Brassicaceae (Tytgat *et al.*, 2013; Wiesner *et al.*, 2013). Since glucosinolate-mediated defence has been overcome by several specialist herbivore species including the butterfly *Pieris brassicae* L. (Wittstock *et al.*, 2004), signals mediating indirect defence mechanisms play an important role for herbivore deterrence in *B. rapa.* In this species, tissue damaged by several caterpillar species can induce volatile organic compounds (VOC), which attract plant mutualists such as parasitoid wasps (Kugimiya *et al.*, 2010; Desurmont *et al.*, 2015). Herbivory also has an indirect effect on a variety of other traits, including floral signals. The onset of flowering (Ågren & Schemske, 1994), flower number (Stowe *et al.*, 2013), flower morphology, and the emission of floral VOCs (Schiestl *et al.*, 2014) can be significantly altered in *B. rapa* plants attacked by herbivores. In some cases, these plastic changes lead to shorter or fewer visits by pollinators (Strauss *et al.*, 1999; Schiestl *et al.*, 2014), which supports the idea of a trade-off between defence and reproduction.

Swift responses of plants to environmental triggers require a fast change in gene expression. A major mechanism involved in this reaction is the reversible methylation of DNA cytidine residues, which is enzymatically catalysed by methyltransferases and often leads to an altered expression of target genes (Holliday & Pugh, 1996; Gutierrez-Arcelus *et al.*, 2015). Stress induced by herbivores can lead to DNA methylation changes in defence-related genes in plants (Grativol *et al.*, 2012). Since partial retention of DNA methylation patterns during meiosis and early embryogenesis allows some of these changes to be passed on to progeny (Calarco *et al.*, 2012), DNA methylation may play a role in priming direct descendants to environmental changes experienced by the parental plants (Hauser *et al.*, 2011; Migicovsky & Kovalchuk, 2013; Heard & Martienssen, 2014). Several cases have been described where at least a part of herbivory-induced DNA-methylation changes were transmitted to offspring along with the observed phenotypic changes (Holeski *et al.*, 2012; Verhoeven & van Gurp, 2012), and one study even recorded an increased resistance to herbivory in the unexposed progeny of stressed plants (Rasmann *et al.*, 2012). Since DNA methylation changes can also dramatically alter floral phenotypes (Cubas *et al.*, 1999; Marfil *et al.*, 2009), methylome alterations induced by herbivory could potentially influence interactions of plants with other insects such as pollinators. However, the role of herbivory-induced DNA methylation changes on pollinator-relevant phenotypic traits has not been thoroughly investigated so far.

In this study, we used methylation-sensitive amplified fragment length polymorphism (MSAP) to screen for methylome changes in *Brassica rapa* plants

subjected to the specialist herbivore *Pieris brassicae* or the plant hormone methyl jasmonate (MeJA). We quantified floral phenotypic changes, compared them with a set of *B. rapa* genotypes demethylated with the DNA methyltransferase inhibitor 5-azacytidine (King, 1995), and assessed the impact of these changes on the pollinator *Bombus terrestris* L. (Rader *et al.*, 2009). Specifically, we hypothesise that a) induction of biotic and chemical defence both lead to tissue-specific DNA methylation changes accompanied by alterations of floral traits in *B. rapa*, b) the observed phenotypic effects are similar to chemically demethylated *B. rapa* plants, and c) phenotypic changes induced by methylome alterations in *B. rapa* are sufficient to change the pollinator attraction.

Results

Alterations of MSAP profiles upon herbivory and methyl jasmonate treatment

Using MSAP, we screened for DNA methylation changes in leaf and flower tissue of herbivore and MeJA-treated *Brassica rapa* R-o-18 plants. The four selective primer combinations amplified a total of 297 fragments between 50 and 500 bp. Of these markers, 295 were susceptible to methylation (proportion of a particular observed *Hpa*II/*Msp*I pattern >5%), and 85 of them were polymorphic in the sampled individuals (29%). A marker was considered polymorphic if both methylated and unmethylated states occurred at least twice across all samples. The remaining two markers were unmethylated, both being polymorphic (S1 Table). With 4.91%, the observed scoring error rate lay within the reported range of ≤ 5% (Bonin *et al.*, 2004). The mean Shannon diversity index for methylation-susceptible loci (0.37) was not significantly higher than for unmethylated loci (0.23, Wilcoxon rank sum test: W=117.5, P=0.363). Results from AMOVA and PCA indicate an inherent methylome difference between leaf and flower tissue within a single individual (Table 1, Fig 1a). In addition, significant induced methylome differentiation was found between all investigated treatments, except between leaves of herbivore and MeJA-treated plants (Table 1). In leaves, both herbivory and MeJA application resulted in a significantly higher proportion of demethylation events across all differentially methylated loci (Fig 1b). In general, treatment effects in flowers were weaker (Fig 1a), and a significant demethylation was only observed for the MeJA treatment (Fig 1b).

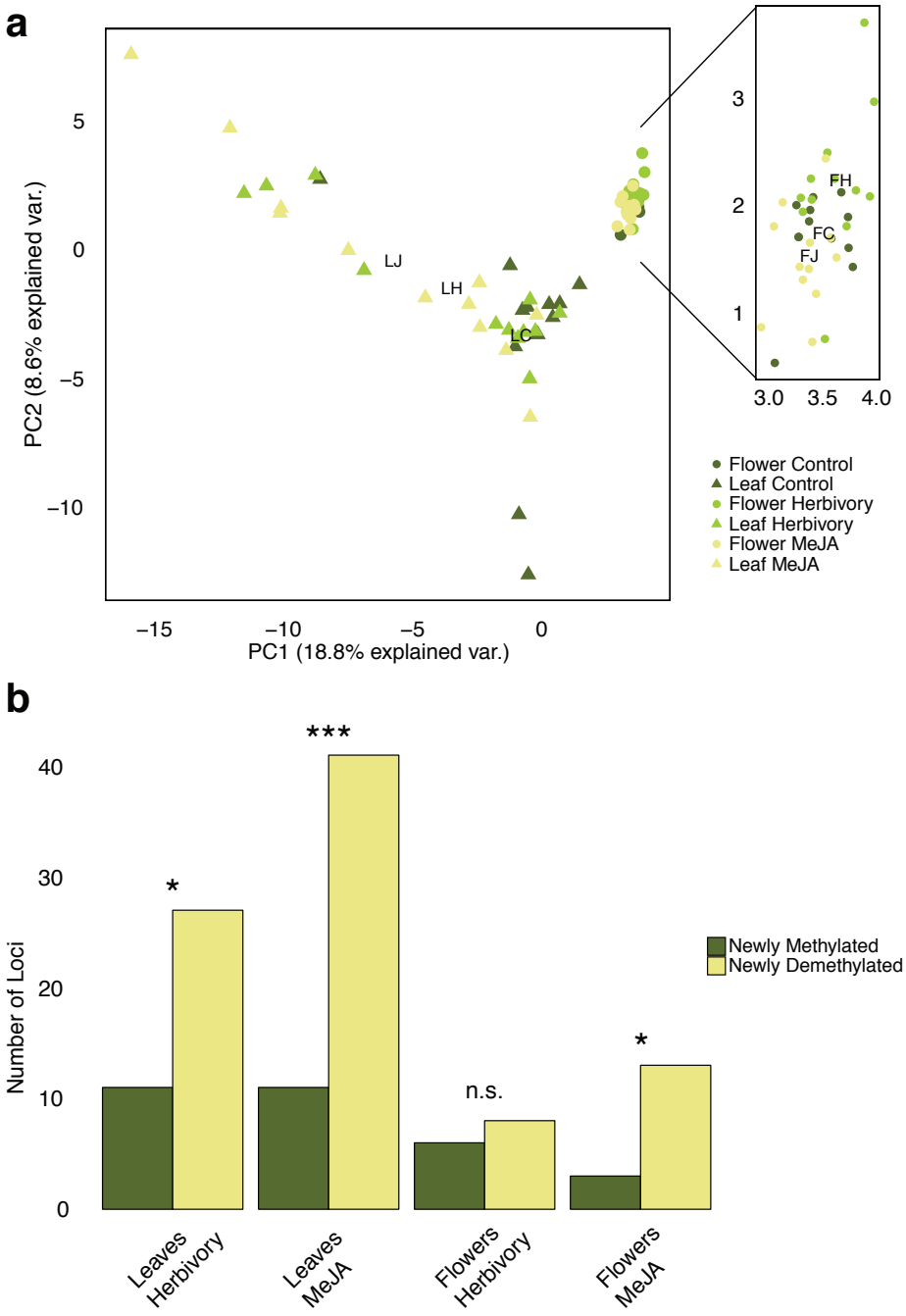

Fig 1 | Treatment effects on DNA methylation (1a): PCA of differentiation in methylation sensitive loci state shows a clear separation of samples from leaves and flowers. Methylome changes upon the different treatments (control, herbivory, and MeJA) were significant in both leaves and flowers, but much more prominent in leaf tissue than in flower tissue. Centroid positions of the control -, herbivory-, and MeJA group are indicated with LC, LH, and LJ for the leaf samples, and with FC, FH, and FJ respectively for the flower samples. **(1b):** Number of loci detected with locus-by-locus AMOVA in both tissues and treatments with either a significant methylation gain (change from an unmethylated to an external, internal, or hypermethylated state as well as change from an external or internal to a hypermethylated state) or methylation loss (change from an external or internal to an unmethylated state as well as change from a hypermethylated to an external, internal, or unmethylated state). Results of the two-sided χ^2-tests: Leaves Herbivory: χ^2=5.921, P=0.015; Leaves MeJA: χ^2=16.173, P<0.001; Flowers Herbivory: χ^2=0.071, P=0.789; Flowers MeJA: χ^2=5.063, P=0.024 (α = 0.05).

Table 1 | Epigenetic differentiation between leaf and flower tissue of Brassica rapa R-o-18 plants after herbivory and MeJA treatment F- and P-values for global and pair-wise AMOVA of methylation-susceptible loci from different tissues (leaves and flowers) of control-, herbivory-, and MeJA-treated plants show a larger epigenetic differentiation between the tissues, and a smaller but still significant epigenetic differentiation between the different treatments except for leaves of herbivory- and MeJA-treated plants. *P* value of significant differentiations in bold (α = 0.05).

Pair-wise AMOVA of meth. markers	**Control Flower**	**Control Leaf**	**Herbivory Flower**	**Herbivory Leaf**	**MeJA Flower**
Control Leaf	F=0.21 **P<0.0001**				
Herbivory Flower	F=0.02 **P=0.0007**	F=0.24 **P<0.0001**			
Herbivory Leaf	F=0.31 **P<0.0001**	F=0.06 **P=0.0244**	F=0.34 **P<0.0001**		
MeJA Flower	F=0.02 **P=0.0008**	F=0.19 **P<0.0001**	F=0.03 **P=0.0006**	F=0.31 **P<0.0001**	
MeJA Leaf	F=0.38 **P<0.0001**	F=0.11 **P=0.0044**	F=0.38 **P<0.0001**	F=0.03 P=0.1153	F=0.37 **P<0.0001**
Global AMOVA of meth. markers	**Deg. of freedom among groups**	**Deg. of freedom within groups**	**Variance among groups**	**Variance within groups**	**F-value**
	5	66	0.02	0.08	F=0.23 **P<0.0001**

Phenotypic effects of herbivory and methyl jasmonate treatments

We compared both floral morphology and floral volatiles (VOC) between the three different *B. rapa* treatment groups. Treatment effects could be measured on overall floral morphology, the entire VOC production, and all different compound classes (S2 Table, Fig 2). Five phenotypic traits as well as eight VOC of all compound classes were significantly reduced (S2 Table). However, herbivory and MeJA had a different impact on morphology and VOC (Table 2, Fig 2): While herbivory caused a decrease of six morphological traits, all but two nitrogenous VOC were unaffected. In MeJA-treated plants, no morphological changes were detected, but the emission of eight VOC distributed over all compound classes was significantly lower. *Z*-3-hexenyl acetate was the only VOC with an increased emission in MeJA-treated plants.

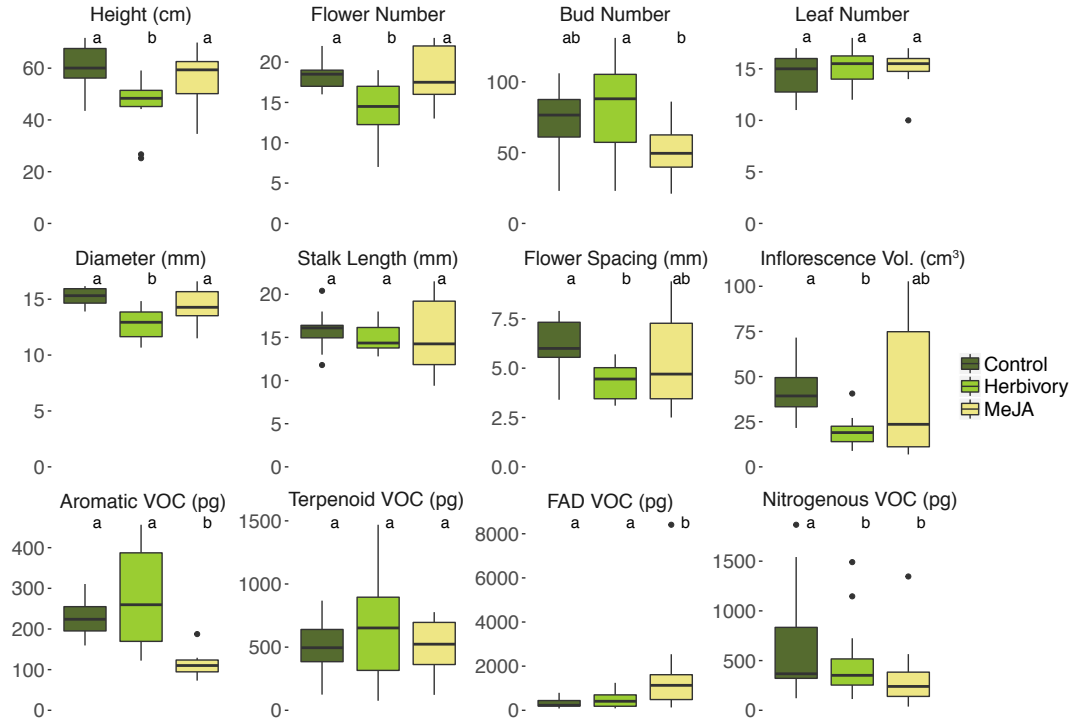

Fig 2 | Treatment effects on morphology and floral volatiles Boxplots showing the effect of the herbivory and MeJA treatment on morphological traits and the emission of the main VOC classes in *B. rapa*. Herbivory led to a decrease in morphological traits (plant height, flower number, diameter, and spacing, and inflorescence volume) and nitrogenous VOC, while MeJA application led to a significant change in the cumulative emission of total aromatic, fatty acid derivatives (FAD), and nitrogenous VOC. Letters (a, b) above boxes indicate different significance groups ($\alpha = 0.05$).

Table 2 | Phenotypic changes of *B. rapa* R-o-18 plants after herbivory and MeJA treatment *Post-hoc* multiple comparisons show a different impact of herbivory and MeJA on the *B. rapa* R-o-18 phenotype. While herbivory treatment mainly decreased morphological traits, the application of MeJA changed floral volatile emission. Emission of the nitrogenous compounds benzylnitrile and methylanthranilate was reduced under both treatments. Arrow up: trait increase, arrow down: trait decrease, dash: no trait change, *P* value of significant changes in bold (α = 0.05).

	Control - Herbivory		Control - MeJA		Herbivory - MeJA	
Plant trait	**change**	***P* value**	**Change**	***P* value**	**change**	***P* value**
Plant Height	↓	**0.002**	-	0.239	↑	**0.027**
Bud Number	-	0.417	-	0.176	↓	**0.044**
Flower Number	↓	**0.006**	-	0.874	↑	**0.006**
Flower Diameter	↓	**< 0.001**	-	0.085	↑	**0.011**
Flower Spacing	↓	**0.041**	-	0.290	-	0.290
Inflorescence Volume	↓	**0.016**	-	0.209	-	0.209
p-Anisaldehyde	-	0.420	↓	**< 0.001**	↓	**< 0.001**
Benzaldehyde	-	0.380	↓	**< 0.001**	↓	**< 0.001**
Methylbenzoate	-	0.970	↓	**< 0.001**	↓	**< 0.001**
Camphor	-	0.753	↓	**< 0.001**	↓	**0.001**
Z-α-Farnesene	-	0.292	↓	**0.014**	-	0.117
Z-3-Hexenyl acetate	-	0.158	↑	**< 0.001**	↑	**0.003**
Benzylnitrile	↓	**0.013**	↓	**< 0.001**	↓	**0.001**
Methylanthranilate	↓	**< 0.001**	↓	**< 0.001**	↓	**0.001**

Genotype-specific effects of DNA demethylation on morphology and VOC

We assessed the effect of 5-azacytidine induced DNA demethylation on morphological traits and VOC production of five *B. rapa* full-sib families. 5-azacytidine is a cytidine analogue, which is incorporated into DNA during replication and inhibits DNA methyltransferases (Jones & Taylor, 1980). Genome demethylation with 5-azaC had a significant, genotype-specific impact on overall plant morphology (S3 Table) as well as the entire floral scent bouquet (S3 Table, Fig 3a). While 5-azaC-treatment decreased all morphological traits (except pollen quantity) across all plant genotypes, the impact of the treatment was highly variable among the different VOC (S4 Table, Fig 3a). Genotype × treatment interactions were present in all morphological traits, but only in aromatic VOC (S4 Table, Fig 3a). Specifically, the results of the pair-wise *t*-tests (Table 3) show considerable morphological variation among genotypes in susceptibility to the 5-azaC-treatment. Genotype as a single factor was significant for all morphological traits except pollen quantity as well as all floral scent compounds (S3 Table), confirming the presence of inherent genotype-specific differences in plant morphology and VOC production.

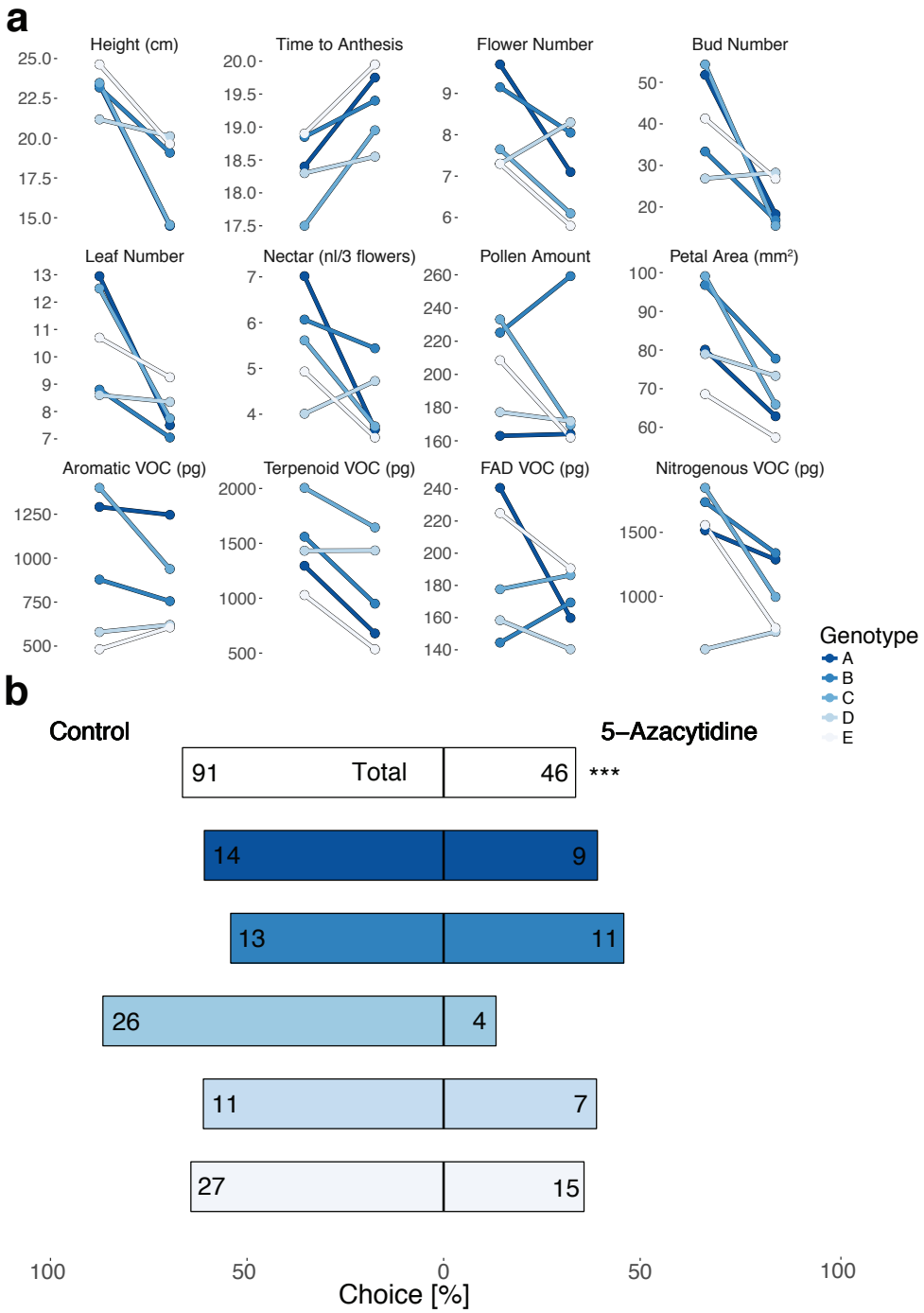

Fig 3 | Effect of DNA demethylation on plant morphology, floral volatiles, and pollinator choice (3a): Plots showing treatment × genotype interactions on morphological traits and floral volatiles (VOC). A comparison of trait divergence between treatment (left points in each line plot) and control group (right points) across all plant genotypes shows pronounced treatment × genotype interactions for all traits (lines connecting both points; e.g. stronger treatment effects in genotype C, and weaker effects in genotype D). Significant treatment effects are indicated in S3 and S4 Table. **(3b):** Barplot showing the choice of *B. terrestris* in the two-choice assays between control and 5-azaC treatment (percentage and total choices) for all plants as well as for individual genotypes. In total, control plants were favoured over 5-azaC-treated plants with 66.4% to 33.6% landings (top bar with significance asterisks, $\alpha = 0.05$). The preference for control plants was dependent on plant genotype and very pronounced in genotype C (bars below).

Table 3 | Phenotypic changes in different genotypes of rapid-cycling *B. rapa* plants after 5-azaC treatment (*post-hoc* multiple phenotypic comparisons) Multiple comparisons show a decreased phenotypic performance in response to genome demethylation in all five different plant families (genotype A-E). However, the five genotypes differ in their susceptibility to the 5-azaC-treatment. While some genotypes (e.g. genotype C) exhibit phenotypic changes in a large number of traits, other genotypes such as genotype D are almost unaffected. Arrow up: trait increase, arrow down: trait decrease, dash: no trait change, *P* value of significant changes in bold (α = 0.05).

	Genotype A		**Genotype B**		**Genotype C**		**Genotype D**		**Genotype E**	
Plant trait	**change**	***P* value**	**change**	***P* value**	**change**	***P* value**	**change**	***P* value**	**change**	***P* value**
Plant Height [cm]	↓	**< 0.001**	↓	**< 0.001**	↓	**< 0.001**	-	0.310	↓	**0.001**
Time to Flowering [d]	↑	**< 0.001**	-	0.109	↑	**< 0.001**	-	0.373	-	**0.017**
Leaf Number	↓	**< 0.001**	↓	**0.022**	↓	**< 0.001**	-	0.597	-	0.158
Bud Number	↓	**< 0.001**	↓	**< 0.001**	↓	**< 0.001**	-	0.705	↓	**0.004**
Flower Number	↓	**0.013**	-	0.140	-	0.107	-	0.140	↓	**0.022**
Flower Petal Area [mm^2]	↓	**< 0.001**	↓	**< 0.001**	↓	**< 0.001**	↓	**0.022**	↓	**0.022**
Plant Petal Area [mm^2]	↓	**< 0.001**	↓	**< 0.001**	↓	**< 0.001**	-	0.338	↓	**0.002**
Nectar Amount [µl]	↓	**< 0.001**	-	0.948	↓	**0.019**	-	0.948	-	0.058
Phenylethyl Alcohol	-	1.000	-	0.196	↓	**0.001**	-	1.000	-	1.000

Attractiveness of demethylated plants to bumblebees

We used dual-choice assays to determine whether the cumulative effect of the observed phenotypic changes in demethylated plants is strong enough to affect the attraction of pollinators. Overall, 5-azaC-treated plants were significantly less attractive to bumblebees (33.6% of all landings, Fig 3b). A comparison between plant genotypes showed a different magnitude of deviation from a 1:1 ratio (genotype A: 39.1% landings, B: 45.8%, C: 13.3%, D: 38.9%, E: 35.7%). However, statistical analysis did not reveal any interaction of treatment and genotype (Fig 3b).

Discussion

Using the model crop plant species *B. rapa*, we investigated the role of DNA methylation in floral signalling in response to herbivory. Our results showed that foliar herbivory of *P. brassicae* caterpillars leads to genome-wide methylation changes not only in the leaves, but also in the undamaged flowers of *B. rapa*. A chemical induction of plant defence resulted in similar demethylation patterns in leaves, but significant differences both in the methylome and phenotype of flowers. The observed methylome changes are thus likely stress-specific and may have the potential to be transmitted to the next generation. Treatment of *B. rapa* with 5-azaC further showed that floral changes observed upon DNA demethylation are correlated with a significant decrease in the attractiveness of the plants to their main pollinator *B. terrestris* (Rader *et al.*, 2009).

DNA demethylation upon induction of plant defence

In plants, an average of around 80% of CpG sites are methylated in a tissue-specific pattern (Gruenbaum *et al.*, 1981; Widman *et al.*, 2014), playing an important role in the regulation of gene activity and immobilisation of transposable elements (Klose & Bird, 2006). The MSAP profiles obtained from the *B. rapa* plants in this study are in agreement with these observations as they show a high proportion of methylated CpG-sites with considerable variation between leaf and flower tissue. Biotic and abiotic stresses have been shown to induce changes in plant methylomes (Grativol *et al.*, 2012; Holeski *et al.*, 2012), and differences in biotic damage could also be linked to variation in DNA methylation (Herrera & Bazaga, 2011). In this study, the stress treatments led to a net shift towards partial or even a complete loss of cytosine methylation. Genome-wide demethylation has been observed in a range of plant systems under various stresses such as high salinity, low temperatures (Choi & Sano, 2007), or viral infection (Mason *et al.*, 2008), and is usually associated with the up-regulation of stress-response genes (Wada *et al.*, 2004). However, the overall picture is far less clear-cut and several other studies have also observed DNA hypermethylation (Kovar *et al.*, 1997; Rico *et al.*, 2014) or no clear trend at all (Tan, 2010). In the genus *Brassica*, it has been shown that leaf herbivory by *Pieris* activates the jasmonate signal pathway (Bruinsma *et al.*, 2009), and spraying *Brassica* plants with MeJA induces defence reactions in leaves (Fritz *et al.*, 2010). This may explain our finding that the methylome in leaf tissue was altered upon treatment, but not significantly different between herbivory and MeJA-treated plants. On the other hand, DNA methylation patterns in flower tissue were different between all three groups, suggesting a stress-specific response to both treatments.

Alterations of floral signals upon herbivory

Fine-tuning of signals such as floral shape, colour, and scent is crucial for the fitness of insect-pollinated plants (Junker & Parachnowitsch, 2015). While it has been shown that herbivory can indeed lead to pollinator-relevant changes in floral signalling (Strauss, 1997; Lucas-Barbosa *et al.*, 2011; Sletvold *et al.*, 2015), results from studies with different plant- and herbivore systems are often very heterogeneous. In *B. rapa*, previous work has not only documented an altered morphology and VOC emission in both leaves and flowers under herbivore attack, but also identified a trade-off between indirect defence and pollinator attraction (Schiestl *et al.*, 2014). Here, we found that herbivory by *P. brassicae* resulted in a net decrease among the measured values of eight traits, many of which are highly relevant to determining plant attractiveness to insects (Wyatt, 1982; Klinkhamer *et al.*, 1989; Worley *et al.*, 2000). However, some of these effects contrast with the findings of Schiestl et al. (Schiestl *et al.*, 2014), where herbivory induced a more pronounced decrease in floral volatiles and an increase in the number of open flowers, which is probably due to different *B. rapa* subspecies used in the two studies (*B. rapa* ssp. *oleifera* in Schiestl et al., and *B. rapa* ssp. *trilocularis* in this study). As reflected by methylome differentiation, effects on floral traits differed significantly between *P. brassicae* and MeJA-treated plants: While the emission of several floral volatiles was more strongly reduced under MeJA treatment, herbivory had a much greater impact on multiple morphological traits. Although it has been shown that plastic responses of *B. rapa* can vary specifically between different types of herbivory (Sotelo *et al.*, 2014), explaining these differences is not straightforward, since a) MeJA may have additional effects beyond plant resistance (Purrington, 2000), b) although the application of 1mM MeJA has been shown to attract parasitoids in *B. oleracea* (Bruinsma *et al.*, 2009), we cannot exclude that this dosage may be on the upper limit of the physiological range, and c) plant reactions to continuous herbivore feeding may be different from reactions to a two-times application of MeJA. A complete understanding of the underlying causes therefore requires additional experiments with varying intensity of both treatments.

Association of DNA demethylation, signalling changes and pollinator choice

Results from this study show that foliar herbivory is associated with both DNA demethylation and floral phenotypic changes. However, it remains challenging to establish a causative link between methylome changes and phenotypic responses since a) methylome structure is not completely independent from genetic variation (Herrera & Bazaga, 2011), and b) other processes such as RNA interference may also induce phenotypic responses in plants (Ahmadovich Bozorov *et al.*, 2012). Several studies have disentangled effects induced by methylome changes from other causes by either comparing methylomes of different organs within single plant individuals (Cubas *et al.*, 1999; Herrera &

Bazaga, 2013), or introduction of DNA methylation changes with chemicals such as 5-azaC (King, 1995; Tatra *et al.*, 2000; Gao *et al.*, 2014). There are some caveats in interpreting the results of 5-azaC experiments: Its action is stochastic and unspecific, and although the application is restricted to a short time during germination, it also incorporates into RNA (Čihák, 1974), which may cause additional effects such as the observed shift in flowering time. However, its impact on DNA methylation is well-documented at the molecular level, and it is useful for assessing the phenotypic impact of DNA demethylation across different genetic backgrounds (Bossdorf *et al.*, 2010). Our finding of demethylation with 5-azaC resulting in a reduced phenotypic expression has been documented in several other studies, including an earlier screen of 5-azaC treated *B. rapa* R-o-18 plants (Amoah *et al.*, 2012). As in the *P. brassicae*-infested plants, the impact of genomic demethylation was more severe on floral morphology than on volatile production. This could reflect that the complex regulation of polygenic morphological traits (Krizek & Fletcher, 2005) may be more exposed to stochastic methylome changes than the regulation of secondary metabolites such as floral scent compounds (see (Dudareva *et al.*, 2013) for a review). While the effect of demethylation on individual traits was consistent among all five genotypes, some genotypes were considerably more affected than others. Bossdorf et al. (2010) (Bossdorf *et al.*, 2010) showed that such differences in demethylation responses are only partially related to genetic distance, which implies that methylome variation is indeed partially independent from genetic differences. As a consequence, the attractiveness of demethylated plants to the pollinator *B. terrestris* tended to be weaker for more affected genotypes, although this relationship was not statistically significant. However, genomic demethylation induced by 5-azaC was sufficient to significantly reduce the overall attractiveness of the treated plants to *B. terrestris.* This result implies that DNA methylation changes *per se* can have a significant impact on plant signalling traits, modulating plant-insect interactions with potential fitness consequences.

In conclusion, our results indicate a strong correlation of DNA methylation states with pollinator-relevant floral traits, which can be selectively altered upon interactions with herbivores. DNA methylation thus has the potential to mediate and interconnect multiple plant-insect interactions through phenotypic plasticity, allowing a quick response to changes in the surrounding insect community. Since flowers are reproductive units, the observed DNA methylation changes may possibly be transmitted to subsequent generations (Calarco *et al.*, 2012). Several studies have indeed shown that stress-induced DNA methylation changes can at least be partially inherited (Holeski *et al.*, 2012; Rasmann *et al.*, 2012).

Material and Methods

Plants and treatments

To minimise the presence of genetic variation that could confound the analysis of DNA methylation states, we used the inbred *B. rapa* ssp. *trilocularis* line R-o-18 in the MSAP experiment (Rusholme *et al.*, 2007). Plants were grown from seeds in 7×7 cm pots with standard soil (Einheitserde Werkverband e.V., Germany) in climate chambers (18 h light, 21°C, 65% relative humidity) with daily watering and no fertilisation. Three days before anthesis, a total of thirty-six plants were randomly assigned to a control-, herbivory-, and MeJA group. Two fifth instar *P. brassicae* larvae were placed each on a mature leaf of plants from the herbivory group and allowed to feed for 24 h. The two infested leaves per plant were encaged in transparent perforated plastic bags to keep the larvae off plant reproductive parts. The treatment of the MeJA group is based on Bruinsma et al. (Bruinsma *et al.*, 2009): The plant defence hormone methyl jasmonate (Sigma Aldrich, Switzerland) was diluted to a 1 mM emulsion in 0.1% Triton X-100 (Sigma Aldrich, Switzerland) and sprayed on vegetative plant parts on two consecutive days (two applications in total). Control plants were left untreated.

The DNA demethylation experiment was conducted with full-sib families generated by manual crossing of rapid-cycling *B. rapa* plants (Wisconsin fast plants, Wisconsin Alumni Research Foundation, WI, USA). Seeds from five of these crossings (genotypes A-E) were treated with 5-azacytidine (5-azaC, Sigma Aldrich, Switzerland) according to King (1998): Seeds were sown on filter paper in petri dishes and soaked in 5-azaC solution (0.05 mM 5-azaC, 0.5 mM 2-(*N*-morpholino)ethanosulphonic acid, pH 6.3). Since 5-azaC treatment delays the flowering time in *B. rapa* (Amoah *et al.*, 2012), the control group was sown two days later on filter paper with 2 ml ddH_2O to ensure simultaneous flowering. Petri dishes were sealed and incubated in the dark at 16°C for 3 days. Seedlings were washed three times with water and transferred to soil. The plants were grown under the same conditions as the plants for the MSAP experiment. All stunted and damaged plants were removed, and the final sample size was balanced to 200 plants (20 plants × 5 genotypes × 2 treatments) by random removal of excess plants.

DNA extraction and MSAP generation

Treated leaves and (untreated) flowers from the R-o-18 plants were collected two weeks after treatment, flash frozen in liquid nitrogen and stored at -80 °C. DNA was extracted using a Qiagen DNeasy Plant Mini Kit (Qiagen, CA, USA) and the manufacturer's protocol, quantified with a Qubit 2.0 fluorometer using a dsDNA-HS assay kit (Thermo Fisher Scientific Inc., CA, USA) and visually checked on a 1.2% agarose gel. Generation of MSAP fragments was performed after Xiong et al. (1999) (Xiong *et al.*) with some modifications. The full protocol is provided in Supplemental Information; enzymes were obtained from New

England Biolabs, MA, USA and from Thermo Fisher Scientific Inc., CA, USA. This protocol uses the enzyme combinations *Eco*RI – *Hpa*II and *Eco*RI – *Msp*I respectively. While *Hpa*II and *Msp*I are isoschizomers recognising the sequence 5'-CCGG-3', *Hpa*II is sensitive to double-stranded methylation of the internal cytosine, and *Msp*I to single-, or double-stranded methylation of the external cytosine (McClelland *et al.*, 1994). Fragments were selectively amplified using four FAM or HEX-labelled primer pairs. One µl selective amplification of each sample was mixed with 10 µl size standard (LIZ 600, Applied Biosystems Inc., CA, USA), diluted 1:100 in Hi-Di-formamide (Applied Biosystems Inc., CA, USA), and denatured for 3.5 min at 92 °C. Fragments were separated on an ABI 3130*xl* sequencer (Applied Biosystems Inc., CA, USA) using the manufacturer's protocols.

MSAP scoring and analysis

The generated MSAP profiles were analysed with GeneMapper v. 4.1 (2009, Thermo Fisher Scientific Inc., CA, USA). Fragments between 50 and 500 bp were included for scoring. Several precautions were taken to ensure reproducibility of the results (Bonin *et al.*, 2004): a) Negative control samples (without DNA) were included in all PCR steps. b) All samples were fully randomised and blindly scored by the same person. c) Loci with electropherogram peaks of less than 100 relative fluorescent units, merged and unclean peaks, peaks occurring in less than 2 samples, and peaks occurring in the negative control samples were removed from the dataset. d) The whole MSAP generation and analysis was repeated from DNA extraction for 17% of all samples (two samples × treatment × tissue). The total scoring error rate was calculated as the ratio of markers scored differently in the replicate samples relative to the total amount of scored markers in the dataset. The MSAP data was analysed with the R package *msap* v. 1.1.4 (Pérez‐Figueroa, 2013): a locus with both *Eco*RI-*Hpa*II, and *Eco*RI-*Msp*I bands present (1/1) was considered unmethylated, a locus with an absent *Eco*RI-*Msp*I band (1/0) externally methylated (single-strand methylation of the external cytosine), and a locus with an absent *Eco*RI-*Hpa*II band (0/1) internally methylated (double or single strand methylation of the internal cytosine). Since the *B. rapa* line (R-o-18) used in this study is highly inbred, loci with absence of both bands (0/0, but present in other individuals in the dataset) were scored as hypermethylated (methylated internal and external cytosines of both strands; see (Lu *et al.*, 2008) for another example). The *msap* package estimates the amount of epigenetic variation based on the Shannon diversity index calculated within each locus. Epigenetic differentiation was computed with principal component analysis (PCA) using the R packages *caret* v. 6.0-68 and *ggbiplot* v. 0.55, and differences between groups were calculated with pair-wise analysis of molecular variance (AMOVA) with 10000 permutations (Pérez‐Figueroa, 2013). In addition, locus-by-locus AMOVA with 10000 permutations was conducted with the R package *mmod* v. 1.3.1 to determine the number of loci

with significant methylation differentiation, and the ratio between newly demethylated and methylated loci was assessed with a two-sided χ^2-test (Schulz *et al.*, 2013).

Morphological and floral volatile analysis

Both phenotypic traits and floral VOCs were collected for all plants individually three days after anthesis. Plant height, flower diameter, and number of leaves, buds, and flowers were recorded. Spacing between flowers and pedicel length of three flowers per plant were measured to assess inflorescence density. For the 5-azaC-treated plants, additional traits including petal surface (4 × π × petal width × petal length), nectar volume and pollen quantity were measured. Anthers from three flowers per plant were collected in 600 µl ddH_2O containing 0.4% Tween 80 (Sigma Aldrich, Switzerland) and pollen was counted on a Cell Lab Quanta flow cytometer with a mercury arc lamp (Beckman Coulter, CA, USA) (Moon *et al.*, 2011).

Flower VOC were collected with non-destructive headspace sorption from 10:00 to 12:00 before the phenotypic measurements. Whole inflorescences were enclosed in glass cylinders treated with Sigmacote (Sigma Aldrich, Switzerland) and sealed with Teflon plates around the peduncle. Clean air was pushed through active charcoal filters into the cylinders at a flow rate of 120 ml min^{-1} for 2 h. Simultaneously, air was pulled out of the cylinders through glass tubes loaded with 20 mg Tenax TA (60/80 mesh, Supelco, Bellefonte, PA, USA) at the same flow rate and duration. Air samples from empty glass cylinders were used as controls. VOC samples were analysed using gas chromatography with mass selective detection (GC-MSD) as described in ref. (Schiestl *et al.*, 2014). Compounds were identified and quantified with a calibrated mass spectral library built on authentic reference standards (Schiestl *et al.*, 2014). Non-identifiable VOC as well as VOC with an amount below the mean air-control level in > 10% of samples were excluded from the dataset. VOC quantities were calculated in pg $flower^{-1}$ l^{-1} sampled air. All analyses were done in the Agilent MSD ChemStation program E. 02.02 (2011).

Bioassays

The attraction of pollinators to 5-azacytidine treated plants was determined in dual-choice bioassays with bumblebees (*Bombus terrestris,* Biobest Group, Belgium). All bioassays were conducted one day after VOC collection to avoid any bias from plant handling. Before the assay, the bees were allowed to forage on untreated *B. rapa* plants from all genotypes for 2 h. Subsequently, one pair consisting of a randomly chosen control, and treated plant of the same genotype was placed with 20 cm distance in a flight cage (2.5 m length, 1.8 m width, 1.2 m height). Bees were released individually in the cage. After the first landing on a flower, the chosen plant was recorded and the bee was removed from the experiment. After a sequence of six visits, all bees were returned to their hive

box, the plant pair was removed and a new pair was installed switching the position of control-, and treated plant. A total of 22 plant pairs and one *B. terrestris* nest box were used in this experiment.

Statistics

Treatment effects were analysed independently for the MSAP and 5-azacytidine experiments. Prior to both analyses, response variables were Box-Cox transformed (Box & Cox, 1964), normality was examined with a Shapiro-Wilk test (Shapiro & Wilk, 1965), and homoscedasticity was assessed using Fligner-Killeen's test (Conover & Iman, 1981). Treatment effects were assessed first on variation of trait classes using multivariate analysis of variance (MANOVA). Morphological variables were combined in one class, and scent compounds were grouped according to chemical classes (aromatics, terpenoids, fatty acid derivatives, nitrogen compounds, and all VOC together), as they partially share biosynthetic pathways (Dudareva & Negre, 2005). For the MSAP experiment, treatment effects within significant MANOVA classes were calculated using a one-way ANOVA, and multiple comparisons between treatments were performed with *post-hoc* pair-wise *t*-tests. For the 5-azacytidine experiment, treatment, genotype and treatment × genotype effects on single variables within significant MANOVA groups were calculated using a two-way ANOVA, and multiple comparisons among plant families were performed with *post-hoc* pair-wise *t*-tests with Bonferroni correction. The effect of the 5-azaC treatment on pollinator attraction was calculated globally using a binomial test, and treatment × genotype effects were assessed with repeated measures ANOVA. All statistical analyses were carried out in *R* v. 3.0.2 (R Development Core Team 2013) with the package *MASS* v. 7.3-35 (Venables and Ripley 2002).

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Supplementary Information

Supplementary Protocol

Sequences of primer and adapters used for the MSAP analysis

Adapter sequences	
*Eco*RI – adapter 1	5’ CTCGTAGACTGCGTACC
*Eco*RI – adapter 2	5’ AATTGGTACGCAGTCTAC
*Hpa*II/ *Msp*I – adapter 1	5’ GATCATGAGTCCTGCT
*Hpa*II/ *Msp*I – adapter 2	5’ CGAGCAGGACTCATGA

Pre-amplification primer	
*Eco*RI – preamp	5’ GACTGCGTACCAATTC
*Hpa*II/ *Msp*I – preamp	5’ ATCATGAGTCCTGCTCGG

Selective primer	
HEX - *Eco*RI – CAA	5’ GACTGCGTACCAATTCCAA
FAM - *Eco*RI – ATG	5’ GACTGCGTACCAATTCATG
*Hpa*II/ *Msp*I – CAA	5’ ATCATGAGTCCTGCTCGGCAA
*Hpa*II/ *Msp*I – TAG	5’ ATCATGAGTCCTGCTCGGTAG

DNA restriction reactions and conditions

***Eco*RI – *Hpa*II restriction**	
NEB buffer 1	10×
*Eco*RI	4 U
*Hpa*II	4 U
DNA	150 ng
ddH_2O	to 40 µl volume

***Eco*RI – *Msp*I restriction**	
NEB buffer 2	10×
*Eco*RI	4 U
*Msp*I	4 U
DNA	150 ng
ddH_2O	to 40 µl volume

Incubation	**Time (min)**	**Temperature (°C)**
Restriction	120	37

Adapter dimerisation reactions and conditions

***Eco*RI Adapter dimerisation**	
NEB buffer 2	10×
*Eco*RI – adapter 1	to 5 µM
*Eco*RI – adapter 2	to 5 µM
ddH_2O	to 30 µl volume

***Hpa*II/*Msp*I Adapter dimerisation**	
NEB buffer 2	10×
*Hpa*II/*Msp*I – adapter 1	to 50 µM
*Hpa*II/*Msp*I – adapter 2	to 50 µM
ddH_2O	to 30 µl volume

Incubation	**Time (min)**	**Temperature (°C)**
Denaturation	5	95

Annealing	120	95 – 25 (touch-down)

Adapter ligation reaction and conditions	
Adapter ligation	
NEB buffer 2	10×
*Eco*RI – adapter (Table S3)	3 µl
*Hpa*II/*Msp*I – adapter (Table S3)	3 µl
T4 DNA ligase	20 U
ATP (100 µM)	0.5 µl
BSA (10 mg/ml)	0.1 µl
ddH_2O	to 10 µl volume
Digested DNA (Table S2)	40 µl

Incubation	**Time (min)**	**Temperature (°C)**
Ligation	180	25
Denaturation	10	65

Pre-selective PCR reaction and conditions	
Pre-selective PCR	
Dream Taq PCR MM	1×
*Eco*RI pre-selective primer	to 35 µM
*Hpa*II/*Msp*I pre-selective primer	to 35 µM
Ligated DNA (Table S4)	2 µl
ddH_2O	to 25 µl volume

Pre-selective PCR cycling	**Time (min)**	**Temperature (°C)**
Denaturation	3	94
20 cycles of:		
Denaturation	0.5	94
Annealing	0.5	60
Elongation	1	72
Final extension	1	72

Selective PCR reaction and conditions	
Selective PCR	
Dream Taq PCR MM	1×
*Eco*RI FAM/HEX labeled primer	to 1.5 µM
*Hpa*II/*Msp*I selective primer	to 4.5 µM
DNA pre-selection (Table S5)	5 µl (diluted 1:20 in 1× TE)
ddH_2O	to 20 µl volume

Selective PCR cycling	**Time (min)**	**Temperature (°C)**
Denaturation	3	94
10 touch-down cycles of:		
Denaturation	0.5	94
Annealing	0.5	65 – 56 (1°C reduction/cycle)
Elongation	1	72
Denaturation	0.5	94
25 cycles of:		
Denaturation	0.5	94
Annealing	0.5	54
Elongation	1	72
Final extension	1	72

Supplementary Tables

S1 Table | Summary of the MSAP analysis on *B. rapa* R-o-18 Used primer pairs, markers between 50 and 500 bp amplified from these primers, scoring error rates (calculated from repeated analysis of 17 % of samples), and frequency, polymorphisms, and calculated Shannon's diversity indices of methylation susceptible and non-methylated markers obtained from leaves and flowers of the total 36 *B. rapa* plants (12 plants per treatment).

			Methylation susceptible markers		**Non-methylated markers**	
Primer pair	**Total markers**	**Scoring error rate**	**Total number**	**Polymorphic**	**Total number**	**Polymorphic**
Eco-ATG / *HM*-CAA	63	4.37%	62	17 (27%)	1	1 (100%)
Eco-ATG / *HM*-TGC	96	5.92%	95	24 (25%)	1	1 (100%)
Eco-CAA / *HM*-CAA	58	3.23%	58	23 (40%)	0	-
Eco-CAA / *HM*-TGC	80	5.42%	80	21 (26%)	0	-
Total	297	4.91%	295	85 (29%)	2	2 (100%)
Shannon's diversity index ± 1 SD			0.37 ± 0.20		0.23 ± 0.03	

S2 Table | Phenotypic differences between *B. rapa* R-o-18 plants under control, herbivory, and MeJA treatment MANOVA results show a general treatment effect on plant morphology and on floral VOC composition, as well as on all chemical compound classes present in the *B. rapa* scent bouquet. ANOVA on individual traits show that the treatments had an effect on 75% of all measured morphological traits and 53% of all quantified VOC.

	MANOVA		**ANOVA**		**Mean ± 1 SE**		
Plant trait	***F* value**	***P* value**	***F* value**	***P* value**	**Control**	**Herbivory**	**MeJA**
Morphological Traits	3.630	**< .001**					
Plant Height [cm]			7.602	**0.002**	60.76 ± 2.45	45.84 ± 2.90	56.16 ± 3.19
Leaf Number			0.974	0.388	14.33 ± 0.62	15.42 ± 0.51	15.00 ± 0.52
Bud Number			3.473	**0.043**	71.25 ± 6.85	81.67 ± 10.34	51.67 ± 5.79
Flower Number			7.083	**0.003**	18.42 ± 0.53	13.92 ± 1.12	18.08 ± 0.98
Flower Diameter [mm]			11.470	**< 0.001**	15.25 ± 0.21	12.84 ± 0.38	14.53 ± 0.45
Flower Spacing [mm]			3.427	**0.044**	6.06 ± 0.40	4.32 ± 0.26	5.26 ± 0.65
Flower Stalk Length [mm]			0.301	0.742	15.81 ± 0.64	14.86 ± 0.44	15.42 ± 1.26
Inflorescence vol. [cm^3]			4.441	**0.020**	41.87 ± 4.36	19.42 ± 2.49	41.70 ± 10.07
VOC [pg / flower l^{-1}]	19.000	**< 0.001**					
Aromatics	4.458	**< 0.001**					
p-Anisaldehyde			34.420	**< 0.001**	16.28 ± 1.40	19.54 ± 2.66	5.63 ± 0.66
Benzaldehyde			21.760	**< 0.001**	172.4 ± 12.8	219.1 ± 29.2	83.81 ± 8.22
Methylbenzoate			24.180	**< 0.001**	21.90 ± 1.58	23.02 ± 2.71	10.03 ± 0.65
Phenylacetaldehyde			3.272	0.051	14.66 ± 2.69	15.45 ± 2.44	8.86 ± 0.92
Phenylethyl alcohol			0.281	0.757	3.87 ± 0.39	3.94 ± 0.47	3.58 ± 0.50
Terpenoids	7.903	**< 0.001**					
Camphor			12.050	**< 0.001**	2.65 ± 0.18	2.61 ± 0.40	1.15 ± 0.16
E-α-Farnesene			0.428	0.655	442.1 ± 49.9	567.6 ± 103.8	472.2 ± 59.0
Z-α-Farnesene			4.718	**0.016**	64.82 ± 9.45	55.33 ± 12.84	26.81 ± 3.05
Fatty acid derivatives	7.162	**< 0.001**					
Z-3-Hexenol			1.219	0.309	133.8 ± 39.9	155.6 ± 34.6	309.9 ± 140.7
Z-3-Hexenyl acetate			12.600	**< 0.001**	136.5 ± 23.8	269.5 ± 62.0	1348.0 ± 504.1
Tetradecane			2.330	0.113	47.85 ± 3.21	53.91 ± 5.53	40.53 ± 3.26
Nitrogenous compounds	12.186	**< 0.001**					
1-Butene-4-Isothiocyanate			2.747	0.079	644.3 ± 171.6	475.4 ± 120.5	312.3 ± 102.4
Benzylnitrile			22.090	**< 0.001**	19.74 ± 2.46	12.14 ± 2.01	4.31 ± 1.06
Indole			1.858	0.172	7.99 ± 1.12	9.76 ± 2.52	1.08 ± 2.97
Methylanthranilate			172.700	**< 0.001**	3.13 ± 0.18	0.64 ± 0.07	0.33 ± 0.05

S3 Table | Phenotypic changes of trait classes in rapid-cycling *B. rapa* plants after 5-azaC treatment
MANOVA results show that both 5-azaC treatment and plant genotype have a significant effect on overall plant morphology and floral VOC emission. Additionally, significant treatment × genotype interactions (t × g) could be observed in both trait groups. Individual MANOVA on different chemical compound classes show that nitrogen-containing compounds do not contribute to the observed treatment effect, and a significant t × g interaction could only be observed for aromatics (*P*-values of significant interactions are in bold).

	MANOVA 5-azaC treatment		**MANOVA genotype**		**MANOVA treatment × genotype**	
Trait class	***F*-value**	***P*-value**	***F*-value**	***P*-value**	***F*-value**	***P*-value**
Morphology	29.264	**< 0.001**	8.169	**< 0.001**	2.977	**< 0.001**
Floral VOC	3.994	**< 0.001**	14.548	**< 0.001**	1.712	**0.001**
Aromatics	4.501	**< 0.001**	20.594	**< 0.001**	1.604	**0.034**
Terpenoids	20.425	**< 0.001**	8.909	**< 0.001**	1.768	0.082
Fatty acid derivatives	5.122	**0.002**	3.703	**< 0.001**	0.614	0.831
Nitrogen-containing compounds	1.767	0.108	14.977	**< 0.001**	1.343	0.127

S4 Table | Phenotypic differences between rapid-cycling *Brassica rapa* plants under control and 5-azacytidine treatment ANOVA on individual traits show that the 5-azacytidine treatment had an effect on 89% of all measured morphological traits and 35% of all quantified VOC. All significant traits were negatively affected, and 9 of these traits showed significant treatment × genotype interactions. Except for pollen quantity, plant genotype had a significant impact on all measured phenotypic traits irrespective of the demethylation treatment. Nitrogen compounds had no significant effect in the MANOVA analysis and were therefore excluded from the analysis. Arrow up: trait increase, arrow down: trait decrease, minus: no trait change, *P* value of significant changes in bold (α = 0.05).

	Mean ± 1 SE		Treatment	ANOVA treatment		ANOVA genotype		ANOVA tre. × gen.	
Plant trait	**Control**	**5-azaC Treatment**	**effect**	***F*-value**	***P*-value**	***F*-value**	***P*-value**	***F*-value**	***P*-value**
Morphological Traits									
Plant Height [cm]	23.16 ± 0.38	17.56 ± 0.44	↓	120.986	**< 0.001**	6.608	**< 0.001**	9.148	**< 0.001**
Time to flowering [d]	18.39 ± 0.10	19.32 ± 0.11	↓	46.875	**< 0.001**	11.118	**< 0.001**	2.864	**0.025**
Leaf Number	10.71 ± 0.30	7.98 ± 0.24	↓	69.038	**< 0.001**	6.407	**< 0.001**	8.655	**< 0.001**
Bud Number	41.52 ± 2.06	21.21 ± 1.34	↓	114.612	**< 0.001**	2.445	**0.048**	13.474	**< 0.001**
Flower Number	8.22 ± 0.25	7.07 ± 0.20	↓	14.520	**< 0.001**	8.123	**< 0.001**	4.237	**0.003**
Flower Petal Area [mm^2]	84.72 ± 1.66	67.45 ± 1.36	↓	105.353	**< 0.001**	25.438	**< 0.001**	7.497	**< 0.001**
Plant Petal Area [mm^2]	704.20 ± 27.31	485.50 ± 19.51	↓	59.871	**< 0.001**	16.286	**< 0.001**	6.803	**< 0.001**
Nectar Amount [µl]	5.52 ± 0.26	4.28 ± 0.24	↓	15.263	**< 0.001**	2.456	**< 0.001**	4.513	**0.002**
Pollen [grains / flower]	67.13 ± 3.79	61.83 ± 3.98	-	2.139	0.145	2.281	0.062	1.017	0.400
VOC [pg / flower l^{-1}]									
Aromatics									
p-Anisaldehyde	24.28 ± 3.81	16.66 ± 2.22	↓	7.104	**0.008**	56.757	**< 0.001**	0.380	0.823
Benzaldehyde	402.90 ± 23.59	397.35 ± 26.88	-	0.355	0.552	4.589	**0.001**	0.913	0.458
Methylbenzoate	124.58 ± 10.44	88.92 ± 6.39	↓	14.407	**< 0.001**	26.106	**< 0.001**	0.922	0.452
Methylsalicylate	58.64 ± 8.52	53.81 ± 5.34	-	0.141	0.708	44.459	**< 0.001**	1.670	0.159
Phenylacetaldehyde	304.12 ± 41.59	266.67 ± 38.67	-	2.515	0.114	51.685	**< 0.001**	1.724	0.146
Phenylethyl alcohol	11.88 ± 1.47	9.94 ± 1.66	↓	9.226	**0.003**	27.316	**< 0.001**	3.158	**0.015**
Terpenoids									
E-α-Farnesene	1406.36 ± 79.12	1010.94 ± 65.89	↓	20.141	**< 0.001**	18.364	**< 0.001**	n/a	n/a
Z-α-Farnesene	58.44 ± 3.09	38.29 ± 2.18	↓	33.420	**< 0.001**	11.754	**< 0.001**	n/a	n/a
Fatty acid derivates									
Z-3-Hexenyl acetate	95.87 ± 9.89	79.00 ± 5.96	↓	4.045	**0.046**	6.650	**< 0.001**	n/a	n/a
Tridecane	10.85 ± 0.67	12.06 ± 1.01	-	0.003	0.958	2.533	**0.042**	n/a	n/a
Tetradecane	82.48 ± 3.45	78.24 ± 3.95	-	2.198	0.140	4.001	**0.004**	n/a	n/a
Nitrogenous compounds									
1-Butene-4-Isothiocyanate	84.94 ± 15.33	66.25 ± 8.82	n/a	n/a	n/a	8.353	**< 0.001**	n/a	n/a
6-Met.-2- Pyridinecarbaldehyde	799.55 ± 80.48	539.91 ± 52.96	n/a	n/a	n/a	8.118	**< 0.001**	n/a	n/a
Benzylnitrile	152.37 ± 15.88	119.82 ± 11.66	n/a	n/a	n/a	31.975	**< 0.001**	n/a	n/a
Indole	166.92 ± 14.07	129.02 ± 12.26	n/a	n/a	n/a	11.124	**< 0.001**	n/a	n/a
Formanilide	9.69 ± 0.73	9.93 ± 0.95	n/a	n/a	n/a	2.476	**0.046**	n/a	n/a
Methylanthranilate	235.31 ± 26.96	155.40 ± 16.04	n/a	n/a	n/a	4.550	**0.002**	n/a	n/a

Chapter II Trans-generational inheritance of herbivory-induced phenotypic changes in *Brassica rapa*

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Abstract

Biotic stress can induce plastic changes in fitness-relevant plant traits. Recently, it has been shown that such changes can be transmitted to subsequent generations. However, the occurrence and extent of transmission across different types of traits is still unexplored. Here, we assessed the emergence and transmission of herbivory-induced changes in *Brassica rapa,* and their impact on interactions with insects. We analysed changes in morphology and reproductive traits as well as in flower- and leaf volatile emission during two generations with leaf herbivory by *Mamestra brassicae* and *Pieris brassicae,* and two subsequent generations without herbivory. Herbivory induced changes in all trait types, increasing attractiveness of the plants to the parasitoid wasp *Cotesia glomerata* and decreasing visitation by the pollinator *Bombus terrestris,* a potential trade-off. While changes in floral- and leaf volatiles disappeared in the first generation after herbivory, some changes in morphology and reproductive traits were still measurable two generations after herbivory. However, neither parasitoids nor pollinators further discriminated between groups with different past treatments. Our results suggest that transmission of herbivore-induced changes occurs preferentially in resource-limited traits connected to plant growth and reproduction. The lack of alterations in plant-insect interactions was likely due to the transient nature of volatile changes.

Introduction

To maximize their fitness, sessile organisms such as plants need to quickly respond to environmental stresses by readjusting the appropriate phenotypic traits (Munns & Termaat, 1986; Puijalon *et al.*, 2008; Franks, 2011; Lange & Lange, 2015). In most cases, these environmentally induced changes are either transient and disappear in the next unstressed generation, or short-term and reset at the latest two generations after induction (Suter & Widmer, 2013; Iwasaki & Paszkowski, 2014). Nevertheless, there are a few studies where the transmission of individual trait changes has been tracked long-term over more than one generation after stress treatment (Whittle *et al.*, 2009; Kathiria *et al.*, 2010; Luna *et al.*, 2012; Rasmann *et al.*, 2012). Despite these findings, the occurrence and relative importance of transient, short-term, and long-term effects has not yet been systematically tested across multiple traits.

Communication with insect visitors is one of the most specific plant-environment interactions. Processes such as the attraction of pollinators or the deterrence of herbivores are mediated by orchestrated signalling of different trait types, e.g. visual perception of morphological traits and olfactory recognition of leaf and flower volatiles (Schiestl & Johnson, 2013; Junker & Parachnowitsch, 2015). Changes such as the appearance of a new herbivore can thus not only lead to simultaneous shifts in many somatic and reproductive trait types (Agrawal, 1998; Agrawal *et al.*, 2012; Johnson *et al.*, 2015), but also create

fundamentally different patterns of trait changes depending on the type of herbivore (e.g. a generalist vs. specialist species) (Ali & Agrawal, 2012). However, measuring such changes is not trivial. In natural populations, genetic, epigenetic, and environmental variation can mask phenotypic effects of induced changes (Johannes *et al.*, 2009; Richards, 2011). On the other hand, studies conducted with (epi-)genetically uniform plants under controlled conditions often focus on particular traits and fail to identify the overall ecological significance of the induced changes (Bossdorf *et al.*, 2008).

One of the best studied plant-insect relationships are the natural interactions of the crop plant *Brassica rapa* L. with both mutualistic and antagonistic insect species: Typically, *B. rapa* deters herbivores via the production of constitutive and inducible glucosinolate defence compounds (Wiesner *et al.*, 2013), but several herbivores such as the specialist *Pieris brassicae* L. or the generalist *Mamestra brassicae* L. are able to detoxify or tolerate these compounds (Wittstock *et al.*, 2004; Poelman *et al.*, 2008). To reduce damage caused by these herbivores, *B. rapa* relies on the attraction of specialized natural enemies such as parasitoid wasps (e.g. *Cotesia glomerata* L.) via herbivore-induced volatile organic compounds (VOCs) (Kugimiya *et al.*, 2010; Gols *et al.*, 2015; Pashalidou *et al.*, 2015). Simultaneously, herbivory has indirect effects on plant morphology and floral VOC production, altering the attractiveness of the plant to pollinators (e.g. *Bombus terrstris* L.) (Strauss *et al.*, 1999; Schiestl *et al.*, 2014). *B. rapa* is thus an ideal system to study the effect of herbivory-induced trait changes on interactions with other insect visitors.

Here, we assessed transient, short-term, and long-term changes in *B. rapa* subjected to leaf herbivory by the specialist *P. brassicae* and generalist *M. brassicae*. Employing a multi-generational approach with minimized standing (epi-)genetic variation and without selection, the objectives of our study were to: 1. measure the induction of trait changes in plant morphology, seed production, and the emission of leaf and flower volatiles under one and two generations of herbivory, 2. determine whether and which of the induced changes are transient or retained one and two generations after herbivory, and 3. assess the effects of these changes on indirect defence and pollinator attraction in bioassays with parasitoids (*C. glomerata*) and pollinators (*B. terrestris*).

Results

Induction and retention of phenotypic changes upon herbivory

Within the four plant generations, four different responses to herbivory were recorded across all twenty-two measured traits (Fig. 1a-d): While seven traits were invariable, herbivory by *Pieris brassicae* and *Mamestra brassicae* induced transient changes in six, short-term effects in three, and long-term effects in five traits. One trait (the VOC methyl salicylate) showed an irregular pattern with only the first and last generation affected (see Table 1, Supplementary Table S1, and Supplementary Fig. S1).

Responses varied strongly between trait types: While transient effects were mostly present among leaf and flower VOCs, short and long-term effects were exclusively recorded among morphological and reproductive traits and completely absent among VOCs. Two generations of herbivory transiently increased mean emission of all three leaf VOCs between 35% and 202% (*M. brassicae*) and between 11% and 66% (*P. brassicae*). Simultaneously, mean emission of four flower VOCs was transiently decreased between 25% and 26% and between 21% and 38%. One generation after treatment, plants still had a 21% (*M. brassicae*) and 11% (*P. brassicae*) smaller petal area per flower, but *M. brassicae*-treated plants produced 25% more seeds. Two generations after treatment, plants had significantly fewer buds (*M. brassicae*: 13%, and *P. brassicae*: 15%), fewer leaves (9% and 9%), and less nectar (33% and 33%). In addition, plants had significantly smaller seeds (7% and 6%), but an increased number of siliques (17% and 27%, see Table 1, Supplementary Table S1, and Supplementary Fig. S1). In plants from both treatment groups, significantly more flowers (open flowers + bud number) developed into siliques two generations after herbivory (two-way ANOVA, F (2,114)=32.21, P <0.001, and *post hoc* Tukey HSD, P (*P. brassicae* - control) <0.001, P (*M. brassicae* - control) <0.001, P (*P. brassicae* - *M. brassicae*) =0.142).

In most cases, induction and direction of trait changes caused by the generalist herbivore *M. brassicae* did not differ from those caused by the specialist *P. brassicae.* However, in *M. brassicae*-treated plants, some morphological and reproductive traits were additionally affected in generation 2 (number of buds and seed viability), and 3 (number of buds, leaves, nectar, and seeds, as well as seed weight). On the other hand, *P. brassicae* treated plants showed an additional transient reduction of the two flower VOCs benzyl nitrile and (*E*)-α-farnesene. Although the background level of the control group was similar across generations for most traits, there were still some noticeable differences: Leaf VOC emission increased in the second treatment generation, and petal size decreased within all four plant generations.

Linear discriminant analysis showed a significant separation of groups across generations and treatments (Wilks' Λ (453,11): 0.242, F = 3.387, $P < 2.2 \times 10^{-16}$). Treatment and control groups were largely separated in the first two

linear discriminant dimensions, which explained 44.0% and 23.3% of the observed group variance. However, while the phenotypes of *M. brassicae* and *P. brassicae* treated plants were indistinct from each other during treatment generations 1 and 2, both variance and separation increased in all treatment groups after treatment in generations 3 and 4. (Fig. 2, Supplementary Table S2).

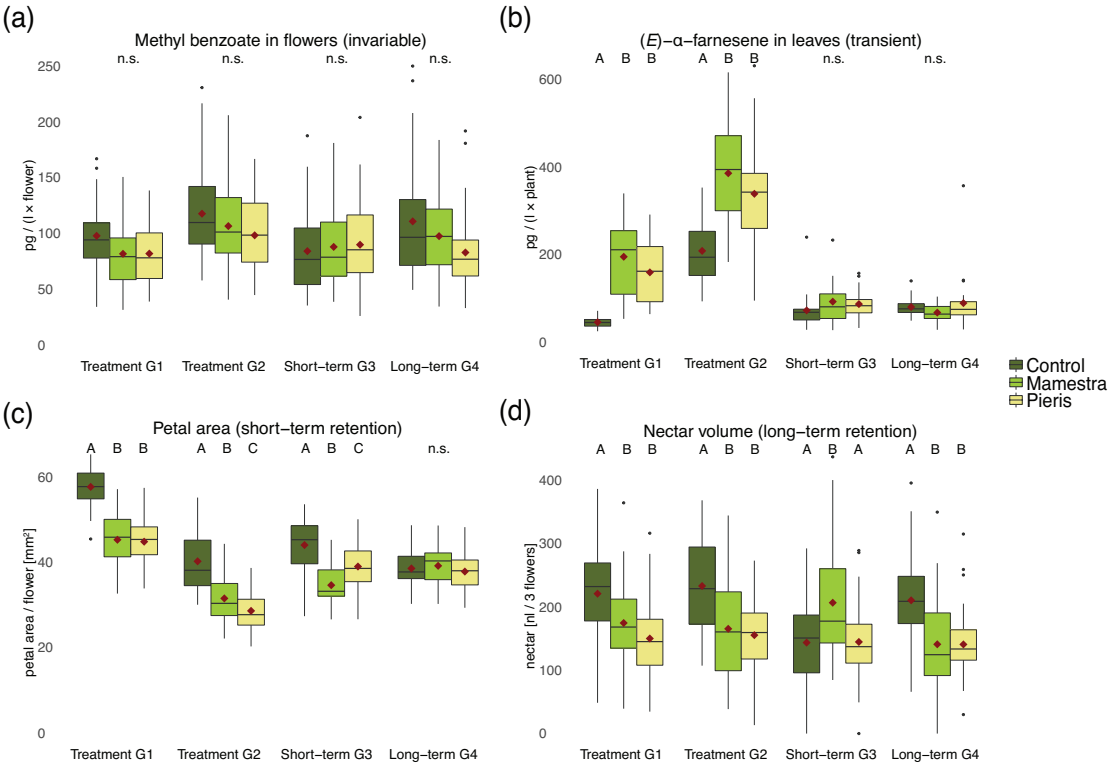

Fig 1 | Examples of traits from all four response types Letters above boxes denote group differences within generation (two-way ANOVA with *post hoc* Tukey HSD), red diamonds denote group means. **(a)**: Invariable response; emission of methyl benzoate. **(b)**: Transient response; emission of (*E*)-α-farnesene. **(c)**: Short-term response; reduction in petal size in both treatment groups. **(d)**: Long-term response; reduction in nectar volume.

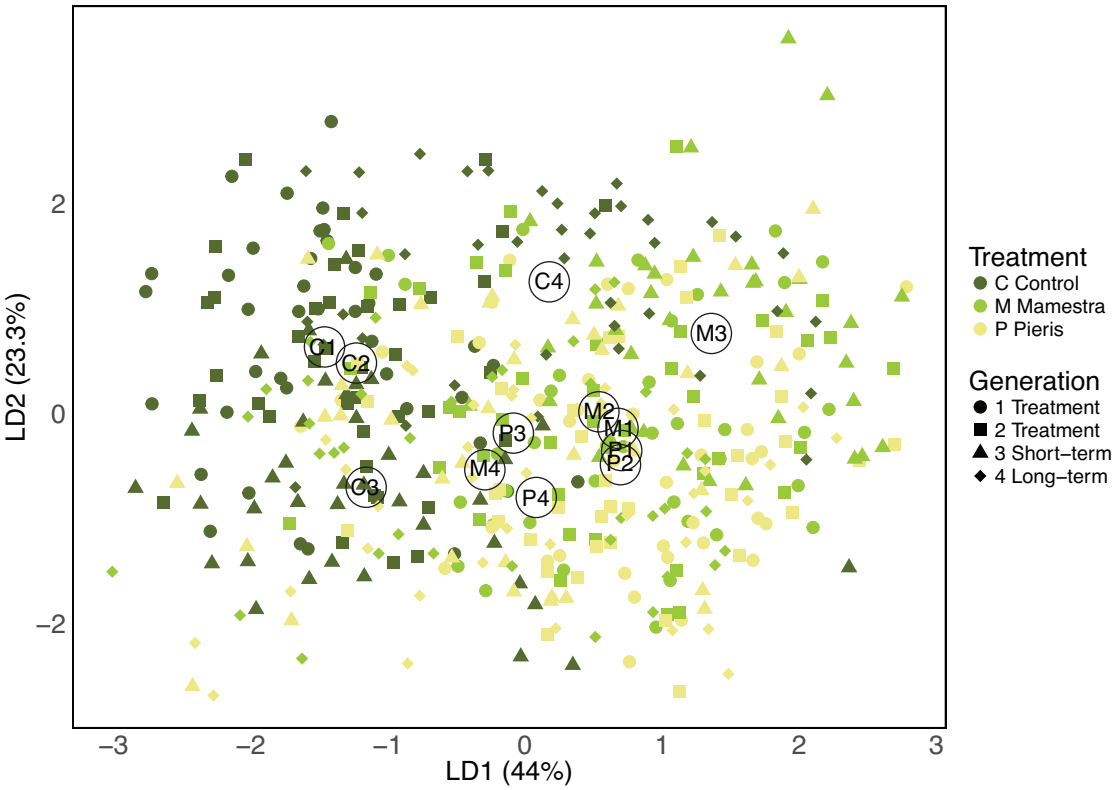

Fig 2 | Result of the linear discriminant analysis (Wilks' Λ (453,11): 0.242, $F = 3.387$, $P < 2.2 \times 10^{-16}$) The first two dimensions (LD1 and LD2) explain 44.0% and 23.3% of the observed group variance (see Supplementary Table S2 for loadings of LD1 and LD2). The analysis was conducted with ranked and z-transformed data of all measured traits except total petal area and leaf VOCs. Letters indicate centroid positions of *Mamestra brassica* (M) and *Pieris brassicae* (P) treated plant groups as well as the control groups (C) of generation 1 - 4.

Table 1 | Summarised MANOVA and ANOVA analyses of trait changes upon herbivory MANOVA of trait types and two-way ANOVA analyses per trait and generation show that fifteen of the twenty-two measured plant traits were significantly altered by the herbivore treatment. While the majority of morphological and reproductive traits showed short or long-term responses two generations after treatment, both leaf and flower VOC were almost exclusively transient. Especially among the reproductive traits, the retained effects in generation (g) 3 and g4 were partially different from the direct responses in g1 and g2, which may be partially due to a decrease in seed viability in the *M. brassicae* group in g2. Significance levels: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. "DF": degrees of freedom within and between groups per generation and trait type. "M": effect on *M. brassicae* group, "P": effect on *P. brassicae* group. Arrow up (↑): increase in trait value, arrow down (↓): decrease in trait value, minus (-): no change in trait value, "n.a.": no ANOVA calculated due to non-significant MANOVA result ($P \geq 0.05$).

	Treatment g1		Treatment g2		Short-term g3		Long-term g4	
	F-value	DF	*F*-value	DF	*F*-value	DF	*F*-value	DF
Morphological traits	**7.79*** **	6, 112	**10.85*** **	6, 110	**7.00*** **	7, 105	**3.34*** **	5, 110
Plant height [cm]	5.19	-	4.45	-	1.61	-	0.36	-
Flower number	2.51	-	3.50	-	3.07	-	1.63	-
Bud number	0.68	-	**9.06****	**M↑**	**12.65*** **	**M↑**	**9.43****	**MP↓**
Leaf number	0.64	-	0.74	-	**18.72*** **	**M↑**	**7.48***	**MP↓**
Nectar [nl / 3 flowers]	**9.82****	**MP↓**	**13.50*** **	**MP↓**	**8.63****	**M↑**	**13.61*** **	**MP↓**
Petal area per flower [mm^2]	**83.59*** **		**47.01*** **	**MP↓**	**39.23*** **	**MP↓**	1.27	-
Total petal area [mm^2]	**27.84*** **	**MP↓**	**21.03*** **	**MP↓**	**9.25****	**MP↓**	2.15	-
Reproductive traits	1.36	6, 112	**6.14*** **	6, 111	**3.51*** **	7, 105	**4.66*** **	5, 110
Silique number	n.a.	-	1.29	-	**8.06****	**MP↑**	**11.79*** **	**MP↑**
Seed weight [mg / 10 seeds]	n.a.	-	5.29	-	**6.56***	**M↑**	**6.20***	**MP↓**
Seed number	n.a.	-	2.92	-	**6.26***	**M↑**	5.76	-
Seed viability [%]	n.a.	-	**6.37***	**M↓**	2.65	-	3.17	-
Leaf VOC [pg/ l]	**8.01*** **	2, 33	**12.76*** **	2, 74	0.68	2, 54	**2.48***	2, 73
1-Butene-4-isothiocyanate	2.38	-	**14.48*** **	**MP↑**	n.a.	-	4.90	-
Benzyl nitrile	**10.99****	**MP↑**	**11.57*** **	**MP↑**	n.a.	-	1.74	-
(*E*)-α-Farnesene	**28.75*** **	**MP↑**	**20.30*** **	**MP↑**	n.a.	-	2.71	-
Flower VOC [pg/flower*l]	**2.95*** **	6, 112	**2.54****	6, 111	**2.65*** **	7, 105	**3.90*** **	5, 110
Benzaldehyde	4.33	-	3.62	-	0.37	-	2.93	-
1,3,5-Trimethylbenzene	4.81	-	3.03	-	1.06	-	3.00	-
Phenylacetaldehyde	3.28	-	0.75	-	1.82	-	1.05	-
Methyl benzoate	4.39	-	3.74	-	0.42	-	5.15	-
Benzyl nitrile	5.42	-	**9.19****	**P↓**	1.33	-	3.46	-
Methyl salicylate	**13.06*** **	**MP↓**	2.98	-	2.71	-	**16.07*** **	**MP↓**
(*Z*)-α-Farnesene	3.89	-	0.10	-	0.05	-	3.27	-
(*E*)-α-Farnesene	**8.73****	**MP↓**	**6.75***	**P↓**	0.86	-	3.97	-

Attractiveness of herbivore-damaged plants to parasitoids and pollinators

The choices made by *Cotesia glomerata* parasitoids were examined with a four-arm olfactometer containing one plant from each treatment and one empty arm. Among plants from generation 1, parasitoids had a significant preference for plants damaged by *P. brassicae,* which is their natural host. Also, none of the wasps chose the empty arm (Fig. 3a). In contrast, no significant choice could be detected among plants from the first and second generation (generations 3 and 4) without treatment, using statistical models either including or excluding the empty arm (Fig. 3a, also see methods). In generations 3 and 4, fewer wasps remained undecided in the central chamber, but some wasps entered the empty arm, which is also an indicator for a lack of a clear signal from one of the arms. In the dual-choice assays, plants subjected to *P. brassicae* herbivory were significantly less attractive to the pollinator *Bombus terrestris* in the treatment generation, but this effect disappeared in the two generations after the treatment. Pollinators did not discriminate between plants subjected to *M. brassicae* herbivory and control plants in all three generations (Fig. 3b).

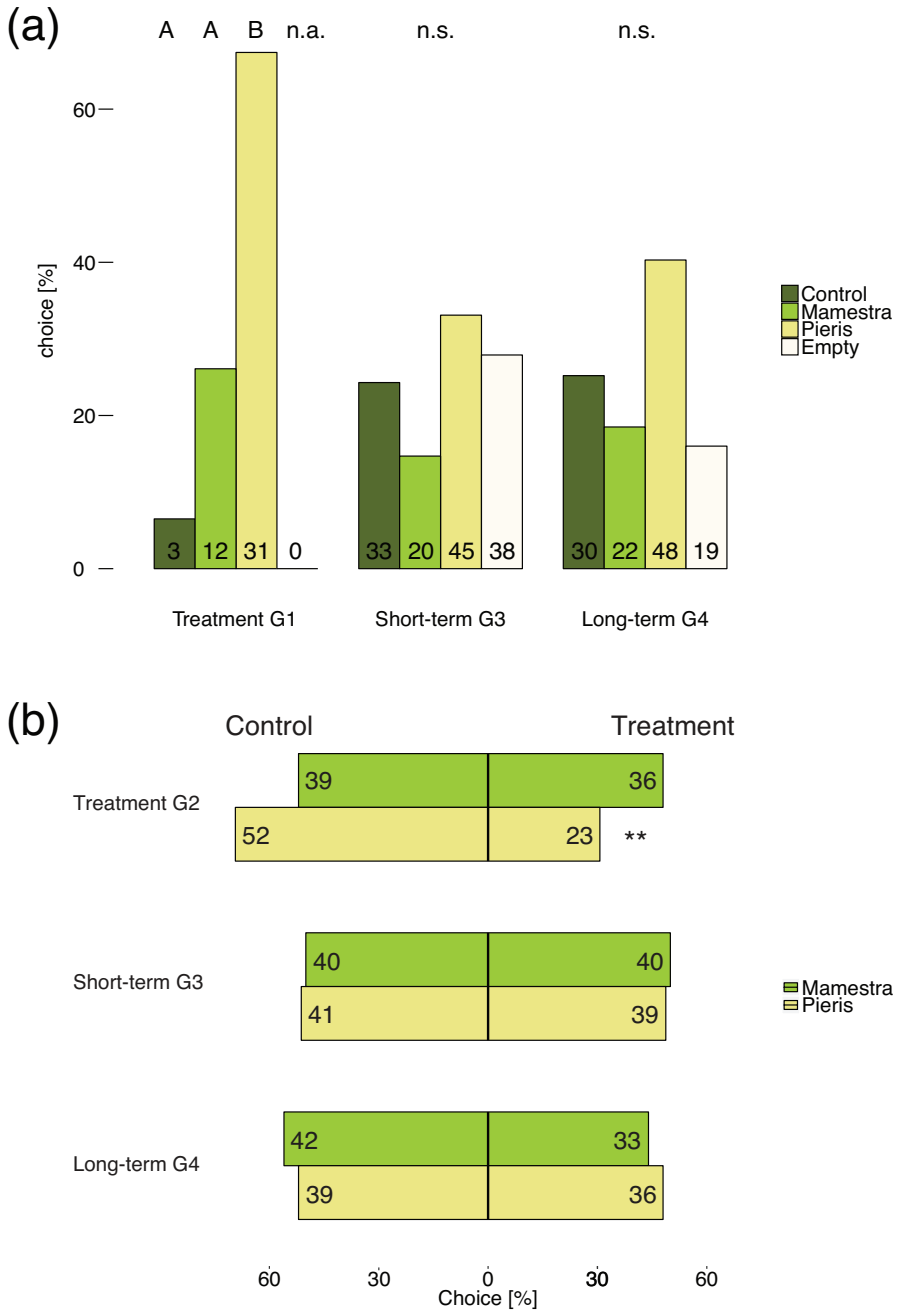

Fig 3 | Results of the bioassays with parasitoids and pollinators (a): Olfactometer tests with the parasitoid wasp *C. glomerata.* Numbers in bars indicate the absolute amount of wasps in this arm, and letters above boxes denote group differences within generation (negative binomial mixed model with *post hoc* Tukey HSD excluding the empty arm due to complete separation, Z (Control-Pieris)=3.806, P (Control-Pieris)<0.001). **(b):** Results of the dual-choice assays with the pollinator *B. terrestris* (binomial test, P=0.001).

Discussion

Using a highly inbred *Brassica rapa* line, we investigated the induction and multigenerational transmission of herbivory-induced phenotypic changes, as well as their impact on plant-insect interactions. Under direct herbivory, changes among plant morphology and volatiles were clearly detectable, reduced pollinator attraction, and induced attraction of parasitoids. After cessation of herbivory, some changes in plant morphology and reproductive traits were retained in one or even two following plant generations, supporting the presence of a trans-generational transmission of some environmentally induced changes. While the potential adaptive nature of these changes is not yet clear, the transient nature of the observed shifts in parasitoid- and pollinator attraction, likely mediated through volatiles, is expected to be advantageous: A retention of these signals would be maladaptive as they would give dishonest cues to parasitoids (Shiojiri *et al.*, 2010) and maintain a trade-off unfavourable for pollinator attraction. We suggest that the action of natural selection on the transmission of induced changes results in a mosaic-like pattern of transient and retained phenotypic alterations.

Previous work has not only demonstrated that herbivory can alter pollinator-relevant traits of *B. rapa* and other plants, but also identified trade-offs between indirect defence and pollinator attraction (Strauss, 1997; Lucas-Barbosa *et al.*, 2011; Schiestl *et al.*, 2014; Sletvold *et al.*, 2015). Altogether, the phenotypic shifts observed in this study are consistent with such a trade-off: Nectar production and petal size, which were both decreased under herbivory treatment, are not only costly in their production (Pyke, 1991; Galen, 1999; Galen *et al.*, 1999) but also have a great impact on pollinator attraction (Conner & Rush, 1996; Kessler *et al.*, 2015). Also, petal size was previously found to be reduced in *B. rapa* under herbivore attack (Strauss *et al.*, 1999). Simultaneously, herbivory increased the emission of all three measured leaf VOCs: 1-butene-4-isothiocyanate is a glucosinolate derivative well-known for its anti-herbivore properties (Agrawal & Kurashige, 2003; Bruce, 2014), and both benzyl nitrile and (*E*)-α-farnesene are reported to be inducible upon herbivory in *B. rapa* (Kugimiya *et al.*, 2010). Studies have shown that 1-butene-4-isothiocyanate as well as benzyl nitrile are attractive to *Cotesia* parasitoids (Kugimiya *et al.*, 2010; Najar-Rodriguez *et al.*, 2015), which may at least partly explain their preference for *P. brassicae*-damaged plants in the first generation of this study. In contrast, floral VOC emission was much less affected, which is congruent with findings of a previous study using another *B. rapa* inbred line (Kellenberger *et al.* (2016), but see Schiestl *et al.* (2014) using wild type *B. rapa* plants). However, the compound (*E*)-α-farnesene has been shown to be positively correlated with reward, and elicits responses in *B. terrestris* olfactory neurons (Knauer & Schiestl, 2015). The observed reduction of this compound together with the smaller petal size may thus explain the reduced attractiveness of *P. brassicae*-infested plants to pollinators. Although it has been shown that herbivores can elicit species-

specific responses in host plants (Agrawal, 2000; Mewis *et al.*, 2006), the response of the *B. rapa* plants in this study was similar for *P. brassicae* and *M. brassicae.* As the two herbivore species have a very similar feeding behaviour, it may be possible that *B. rapa* cannot distinguish between *P. brassicae* and *M. brassicae* based on oral secretion alone.

So far, persistence of herbivore-induced phenotypic changes across generations has been documented mainly in the context of direct defence: It has been shown that herbivory can induce trans-generational changes in defensive plant traits such as trichomes (Holeski, 2007), cyanides (Ballhorn *et al.*, 2016), glucosinolates (Rasmann *et al.*, 2012), and other phytochemicals (Holeski *et al.*, 2013). However, systematic screens also including non-defensive traits and plant volatiles have hardly been conducted. Trans-generational effects observed in this study were restricted to morphological and seed traits, with most of them showing a reduced performance. As visible in different trait levels of the control group across generations, unknown physiological or environmental factors also led to inter-generational variation in the overall performance of the plants, which might have co-influenced the magnitude of trait changes. However, many of the affected traits can be considered rather costly, and experiments with *Pieris* have shown a similar reduction in biomass (Rasmann *et al.*, 2012) and seed size (Agrawal, 2002) among descendants of herbivory damaged Brassicaceae plants. The observed reduction could thus be either a negative effect resulting from the inferior performance of the treated ancestral plants, or an adaptive reallocation of resources (Herman & Sultan, 2011). While our study did not look at direct defence mechanisms, it is plausible that resources might also be reallocated to glucosinolate biosynthesis in the progeny. In the last years, molecular mechanisms have been described which could provide a framework for the inheritance of such induced changes, including maternal effects (Roach & Wulff, 1987; Miao *et al.*, 1991; Wolf & Wade, 2009) and epigenetic mechanisms such as DNA methylation, histone modifications, or RNAi (Bossdorf *et al.*, 2008; Hauser *et al.*, 2011; Holeski *et al.*, 2012).

In the generations after treatment, changes in plant volatiles, which are important signals for insect attraction, were not transmitted to offspring after treatment. Also, both parasitoids and pollinators did not discriminate among the progeny of the herbivore-treated plant groups. Since a constitutive attraction of parasitoids would be a dishonest "cry wolf " signal (Shiojiri *et al.*, 2010), it can be assumed that the plastic nature of signalling effective to parasitoids is adaptive. This may also be the case for the reduced attraction of pollinators, which should be reset as soon as trade-offs between defence and attraction are released. Although our study did not show whether any of the retained phenotypic changes have an adaptive value, it provides some hints on possible fitness-relevant properties: Unlike in control plants where later-developing flowers usually do not produce siliques anymore, seed production in progeny of herbivore-treated plants is distributed over the whole flowering time and among

the majority of flowers. This interesting shift in resource allocation should be further evaluated in future studies.

Material and Methods

Plants and treatments

To minimize the presence of standing genetic and epigenetic variation that could mask *de novo* phenotypic changes, we used seeds from a single individual of the self-fertilizing inbred *Brassica rapa* line IMB 211 (Iñiguez-Luy *et al.*, 2006) for the first generation (Fig. 4, see (Whittle *et al.*, 2009) for a similar setup). Plants were grown in 7×7 cm pots with standardized soil (Einheitserde Werkverband e.V., Germany) and randomized daily in a climate chamber (24 h light, 21°C, 65% relative humidity, daily watering). Fourteen days after germination, a total of 150 plants were randomly assigned either to a control, *Mamestra brassicae*, or *Pieris brassicae* group (Fig. 4). Two first instar *M. brassicae* or *P. brassicae* larvae obtained from an in-house breeding were placed each on the newest (fourth) mature leaf of plants from the corresponding group and allowed to feed for seven days (ca. 1-2 days before anthesis; control plants were left untreated). Aiming at damage of ca. 25% of total leaf tissue per plant, inactive larvae and larvae reaching the third instar were replaced with new ones as needed. Plants with damaged reproductive parts (flower stalks and buds) were removed, and the final sample size was balanced to 120 plants (40 plants per group, Fig. 4). Seven days after herbivore removal, all plants were transferred to a greenhouse table under an insect net and kept under the same conditions as above until seed ripening.

For the second generation, ten seeds from each of the 120 plants were sown, germination rate was recorded from these seeds, and three seedlings per parent were chosen, resulting in a total of 360 plants (3×40 plants per group, Fig. 4). Plants received the same conditions and treatments as in generation 1. After herbivore removal, plants with damaged reproductive parts (flowers and stalks) were excluded, and one of the remaining descendants per parent was randomly selected to again balance the sample size to 120 plants (40 plants per group). The other plants were used for pollinator bioassays (see below). After generation 2, herbivore treatment was ceased and the plants were grown for two more generations (generations 3 and 4) under the same environmental conditions and propagation regime (Fig. 4).

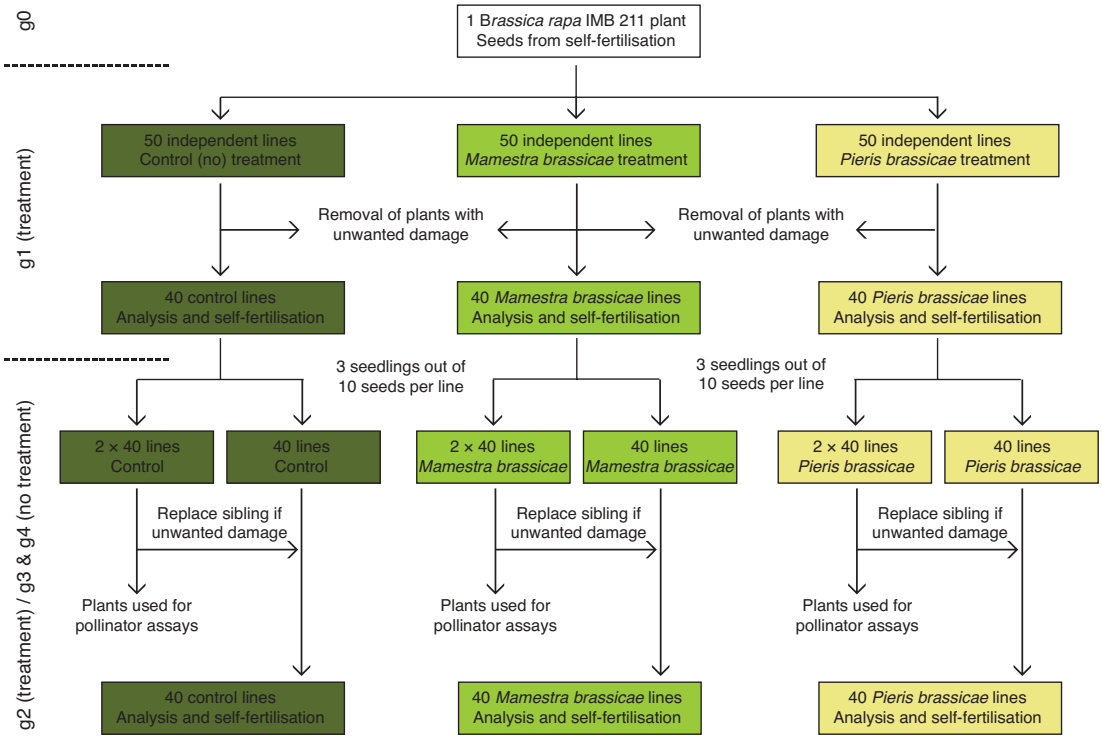

Fig 4 | Propagation scheme showing the initial plant (g0), the two generations with herbivore treatment (g1 and g2), and the two generations without herbivory (g3 and g4) of this experiment.

Morphological analysis

Phenotypic analysis was conducted at four different time points: 1. leaf VOCs were collected from 20 randomly selected plants per group 48 h after the onset of herbivory. 2. flower VOCs as well as all phenotypic measurements (except nectar and seed traits) were collected from all plants individually three days after anthesis. Plant height, mean petal surface of three flowers (4 petals × π × 0.5 petal width × 0.5 petal length), and number of leaves, buds, and open flowers were recorded. 3. mean nectar production of three flowers was measured for all plants with 5 µl glass capillaries six days after anthesis. 4. number of siliques and mean seed weight of ten seeds were recorded from all plants after seed ripening. Total seed weight was further used to estimate the total amount of seeds per plant.

Volatile analysis

Volatiles were collected from leaves and inflorescences with non-destructive headspace sorption. Whole plants (leaf VOC) or inflorescences (flower VOC) were enclosed in Sigmacote-treated glass cylinders (Sigma Aldrich, Switzerland) and sealed with aluminium foil (leaf VOC) or Teflon plates around the peduncle (flower VOC). VOCs were sampled by simultaneously pushing clean air through active charcoal filters into the cylinders, and sucking air out through glass tubes loaded with 20 mg Tenax TA (60/80 mesh) at a flow rate of 100 ml min^{-1} for 2 h. Background VOC levels were determined with samples from empty glass

cylinders. Analysis of VOC samples was performed using gas chromatography with mass selective detection (GC-MSD) as described in Schiestl *et al.* (2014). Identification and quantification of compounds was achieved with a mass spectral library built on calibration curves using three to five different concentrations of authentic reference standards (Schiestl *et al.*, 2014). Non-identifiable VOC as well as VOC with an amount below the mean background level in > 10% of samples were excluded from the dataset. VOC quantities were converted to pg flower^{-1} l^{-1} sampled air. All analyses were done using the Agilent MSD ChemStation program E. 02.02 (2011).

Bioassays with parasitoids

Between 24 and 48 h after the onset of herbivory treatment, five (in generations 1 and 4) or six plants (in generation 3) each from the control and treatment groups were randomly selected and brought to a laboratory at the University of Neuchâtel, Switzerland. Plants from generation 2 were mechanically damaged during transport and thus could not be used for this assay. The herbivores were removed and the soil was covered with aluminium foil around the hypocotyl. One plant per treatment group (= one set of three plants) was randomly placed in a clean four-arm olfactometer technically identical to the one described in Turlings *et al.* (2004), with a pot containing aluminium foil covered soil in the empty fourth arm. Clean air was pushed through each arm with a flow rate of 0.7 l min^{-1}, converging in a central chamber, in which five mated female *Cotesia glomerata* parasitoid wasps were released. All wasps were obtained from an in-house breeding. After 30 min, the wasps were removed and their choice was recorded; wasps residing in the central chamber were counted as undecided. This procedure was repeated five times to a total of 5×5 wasps per plant set. Afterwards, the olfactometer was cleaned with acetone and pentane and a new plant set was installed.

Bioassays with pollinators

The attraction of pollinators to herbivory-treated plants was determined in dual-choice bioassays with bumblebees (*Bombus terrestris,* Andermatt Biocontrol, Andermatt, Switzerland). The assays were conducted with excess plants from generations 2, 3, and 4 (Fig. 4, see above) five days after onset of anthesis, corresponding to six to seven days after herbivore removal. Before the assay, the bees were allowed to forage on untreated *B. rapa* plants for 2 h. Subsequently, one pair consisting of a randomly chosen control, and a either an *M. brassicae* or a *P. brassicae* treated plant was placed with 20 cm distance in a flight cage (2.5 m length, 1.8 m width, 1.2 m height). Bees were released individually in the cage. After the first landing on a flower, the chosen plant was recorded and the bee was removed from the experiment. After a sequence of five visits, all bees were returned to their hive box, the plant pair was removed and a new pair was installed switching the position of control-, and treated plant. A total of 15 plant pairs per herbivore group and generation were used in this experiment.

Plant trait statistics

Prior to the statistical analysis, all response variables were Box-Cox transformed (Box & Cox, 1964) and tested for normality and homoscedasticity with a Shapiro-Wilk test (Shapiro & Wilk, 1965), and Fligner-Killeen's test (Conover & Iman, 1981) respectively. Response variables were assigned to a morphological traits class, reproductive traits class, leaf VOC class, and floral VOC class. Effects on trait classes were analysed for each plant generation individually with a two-way MANOVA with treatment and sampling day (resulting from time to anthesis) as explanatory variables. Treatment effects on traits within significant MANOVA classes were calculated using a two-way ANOVA with holm adjustment of all *P*-values within this plant generation, and comparisons between treatments were performed with *post hoc* Tukey HSD tests. Finally, all response variables (except total petal area and leaf VOCs) were ranked within generation, z-transformed across all generations, and overall treatment effects were assessed with linear discriminant analysis (LDA). For classification, both generation and treatment were combined in one grouping factor (4 × 3 = 12 classes), and the discriminatory power was tested using Wilks' Λ. Statistical analyses were carried out in *R* v. 3.0.2 (R Development Core Team 2013) with the packages *MASS* v. 7.3-47 (Venables and Ripley 2002) and *caret* v. 6.0-76 (Kuhn 2008).

Bioassay statistics

As suggested by Turlings *et al.* (2004), we analysed preferences of *C. glomerata* wasps with a negative binomial mixed model adjusting for zero inflation and overdispersion. Treatment was included as fixed effect, and the interaction between treatment and plant group as random effect. In generation 1, none of the wasps chose the empty arm, which lead to complete separation of the random term in the model. We thus restricted our analysis to the three arms containing control and treated plants to enable a comparison between plant generations. However, we additionally fitted models including all four arms for generations 3 and 4 to assess choice difference between all arms within those generations. For all models, comparisons between treatments were performed with *post hoc* Tukey HSD tests. The effects of *P. brassicae* and *M. brassicae* herbivory on pollinator attraction were calculated using a binomial test. Statistical analyses were carried out in *R* with the package *glmmADMB* v 0.8.3.3 (Bolker et al. 2012).

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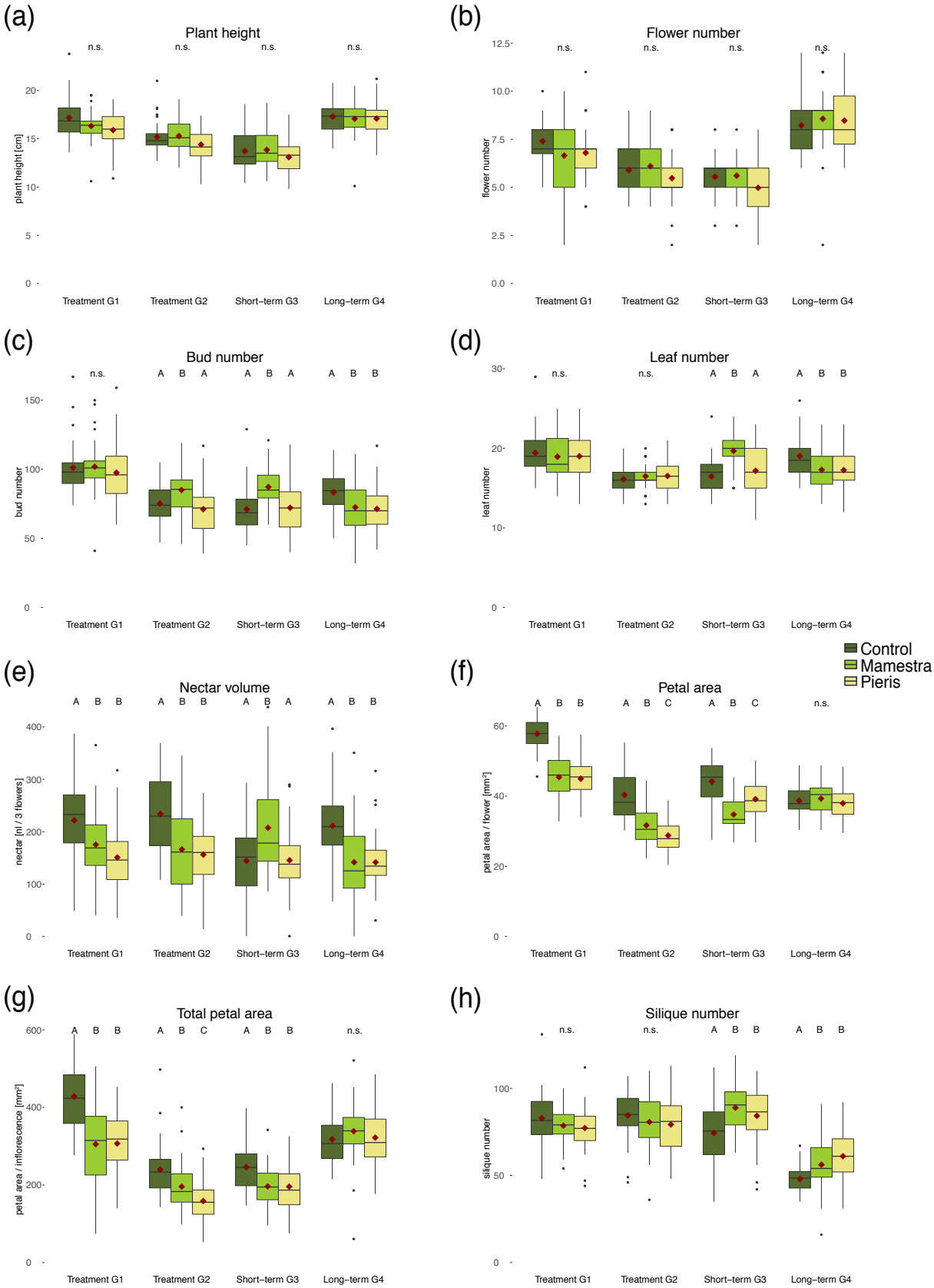
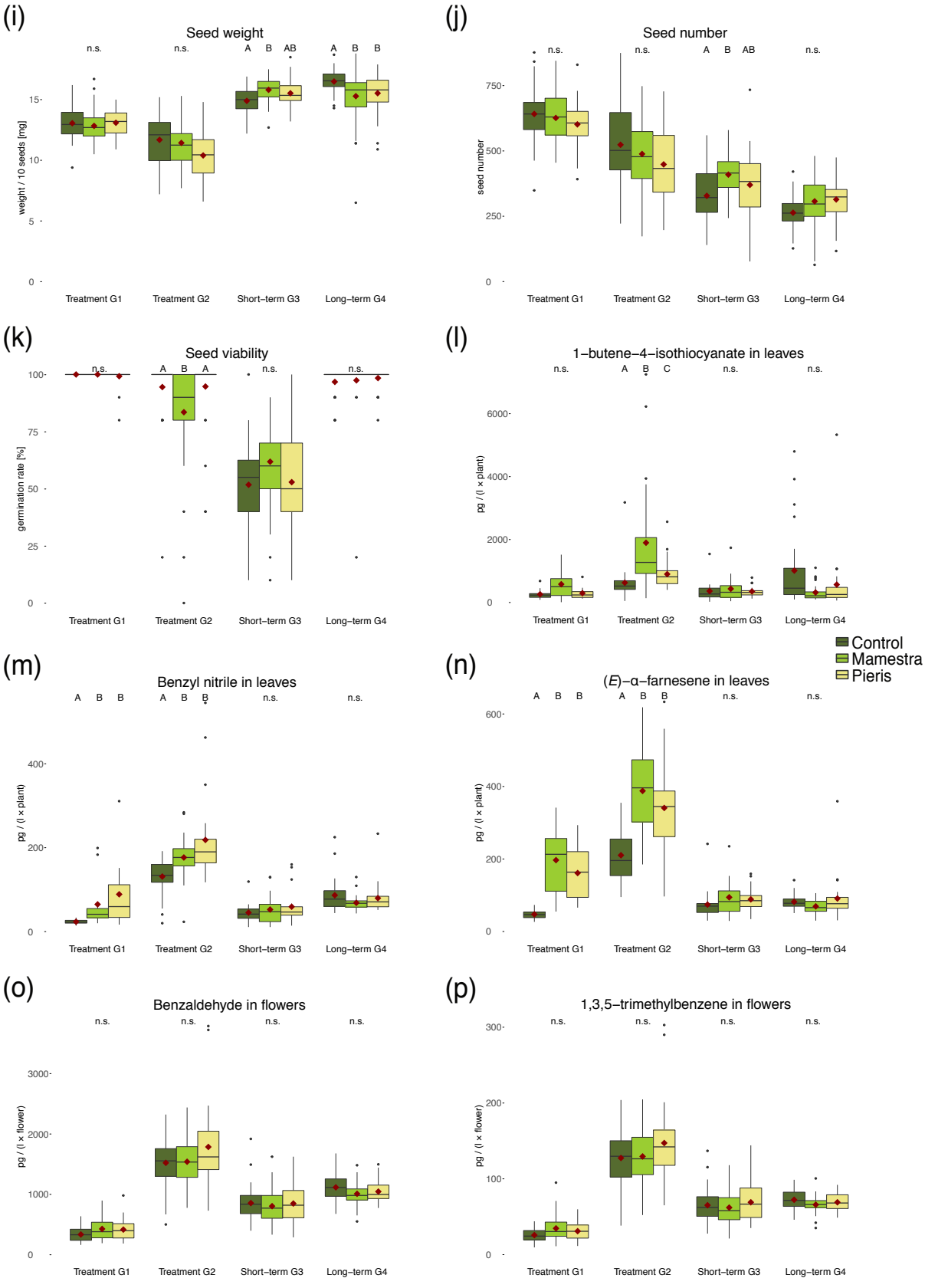
Supplementary Information

Supplementary Figures




(i)
Seed weight
weight / 10 seeds [mg]
n.s.
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(k)
Seed viability
germination rate [%]
n.s.
A B A
n.s.
n.s.
(l)
1−butene−4−isothiocyanate in leaves
pg / (l × plant)
n.s.
A B C
n.s.
n.s.
(m)
Benzyl nitrile in leaves
pg / (l × plant)
A B B
A B B
n.s.
n.s.
(n)
(E)−α−farnesene in leaves
pg / (l × plant)
A B B
A B B
n.s.
n.s.
Control
Mamestra
Pieris
(o)
Benzaldehyde in flowers
pg / (l × flower)
n.s.
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n.s.
n.s.
(p)
1,3,5−trimethylbenzene in flowers
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Treatment G1
Treatment G2
Short−term G3
Long−term G4

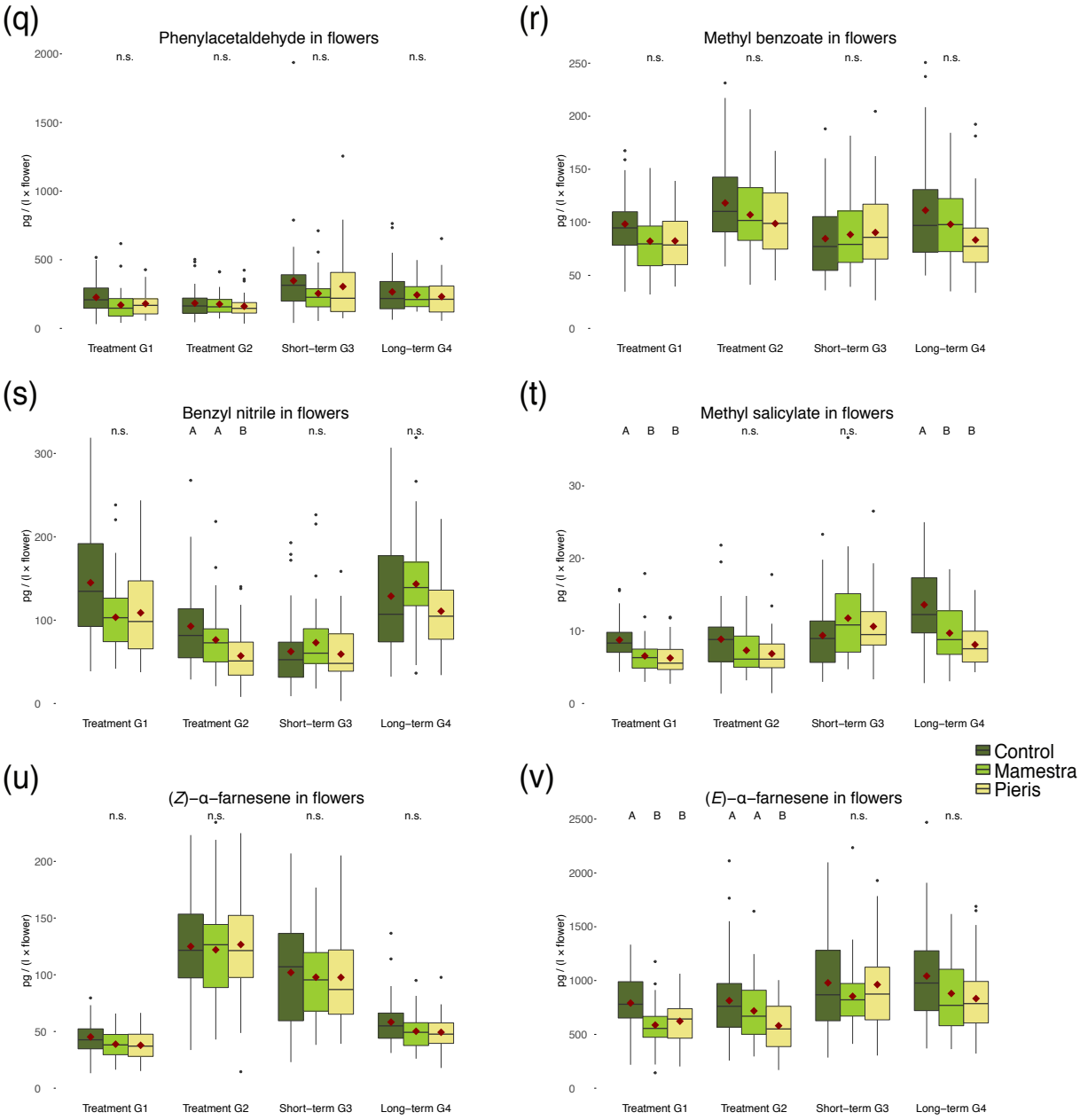

Figure S1. Boxplots showing treatment effects on all measured phenotypic traits across all four plant generations (Red diamonds denoting group means). (a) to (g) show morphological traits, (h) to (k) show reproductive traits, (l) to (n) show leaf VOC, and (o) to (v) show flower VOC. Letters above bars indicate significant trait differences between treatment groups in this plant generation (two-way ANOVA with *post hoc* Tukey HSD).

Supplementary Tables

Table S1. Trait means and standard errors Both mean and ± 1 standard error (SE) are shown for each treatment group per trait (all morphological traits as well as all leaf and floral VOC) and generation (both g1 and g2 with direct treatment as well as g3 with short retaining effects, and g4 with long retaining effects)

	Treatment g1 Mean ± 1SE			Treatment g2 Mean ± 1SE			Short retaining g3 Mean ± 1SE			Long retaining g4 Mean ± 1SE		
	Control	*Mamestra*	*Pieris*	Control	*Mamestra*	*Pieris*	Control	*Mamestra*	*Pieris*	Control	*Mamestra*	*Pieris*
Morphological traits												
Plant height [cm]	17.2 ± 0.3	16.3 ± 0.2	15.9 ± 0.3	15.2 ± 0.3	15.3 ± 0.3	14.4 ± 0.3	13.7 ± 0.3	13.9 ± 0.3	13.1 ± 0.3	17.3 ± 0.3	17.1 ± 0.3	17.1 ± 0.3
Flower number	7.4 ± 0.2	6.7 ± 0.3	6.8 ± 0.2	5.9 ± 0.2	6.1 ± 0.2	5.5 ± 0.2	5.6 ± 0.2	5.6 ± 0.2	5.0 ± 0.2	8.2 ± 0.2	8.6 ± 0.3	8.5 ± 0.2
Bud number	101.1 ± 2.9	101.9 ± 3.0	97.6 ± 3.3	75.2 ± 2.2	85.0 ± 2.5	71.1 ± 2.8	71.1 ± 2.5	87.2 ± 2.3	72.2 ± 2.8	83.4 ± 2.5	72.5 ± 2.8	71.3 ± 2.5
Leaf number	19.5 ± 0.5	19.0 ± 0.5	19.0 ± 0.5	16.1 ± 0.3	16.5 ± 0.2	16.5 ± 0.3	16.5 ± 0.4	19.7 ± 0.4	17.2 ± 0.5	19.0 ± 0.4	17.3 ± 0.4	17.3 ± 0.5
Nectar [nl/3 flowers]	221.7 ± 12.2	175.4 ± 11.4	150.9 ± 10.1	233.9 ± 11.7	166.3 ± 13.2	156.2 ± 10.6	144.4 ± 10.8	207.5 ± 14.4	145.5 ± 10.2	211.3 ± 11.3	141.8 ± 11.2	141.6 ± 9.0
Petal area/flower [mm^2]	57.8 ± 0.7	45.4 ± 0.9	45.0 ± 0.9	40.4 ± 1.1	31.7 ± 0.9	28.8 ± 0.8	44.2 ± 0.9	34.8 ± 0.7	39.1 ± 0.9	38.7 ± 0.7	39.3 ± 0.7	38.0 ± 0.8
Total petal area [mm^2]	428.2 ± 12.7	305.2 ± 16.0	307.0 ± 11.7	242.5 ± 11.8	198.1 ± 9.9	163.3 ± 10.5	245.7 ± 9.7	196.2 ± 8.3	195.7 ± 9.9	317.9 ± 10.2	338.6 ± 12.2	322.1 ± 11.8
Reproductive traits												
Silique number	82.9 ± 2.3	78.6 ± 1.5	77.2 ± 2.0	84.5 ± 2.3	80.7 ± 2.4	79.2 ± 2.7	74.3 ± 2.8	88.9 ± 2.3	84.2 ± 2.6	48.0 ± 1.3	56.1 ± 2.2	61.0 ± 2.3
Seed weight [mg/10 seeds]	13.1 ± 0.2	12.8 ± 0.2	13.1 ± 0.2	11.7 ± 0.3	11.4 ± 0.3	10.4 ± 0.3	14.9 ± 0.2	15.8 ± 0.2	15.5 ± 0.2	16.5 ± 0.1	15.3 ± 0.4	15.5 ± 0.3
Seed number	641.6 ± 16.5	626.3 ± 14.4	601.7 ± 13.0	523.6 ± 23.0	488.4 ± 19.9	448.5 ± 22.5	328.3 ± 15.1	409.4 ± 12.6	370.1 ± 20.3	263.6 ± 9.9	307.2 ± 15.1	314.3 ± 13.8
Seed viability [%]	100.0 ± 0.0	100.0 ± 0.0	99.2 ± 0.6	94.5 ± 2.3	83.5 ± 3.6	94.7 ± 2.5	51.8 ± 3.3	61.8 ± 2.9	52.9 ± 4.0	96.8 ± 1.0	97.4 ± 2.1	98.4 ± 0.7
Leaf VOC [pg/ l]												
1-Butene-4-isothiocyanate	261.7 ± 47.5	580.9 ± 128.7	295.4 ± 57.2	629.1 ± 114.6	1898.5 ± 334.7	901.2 ± 91.3	363.2 ± 75.2	434.5 ± 91.7	351.2 ± 38.8	1012.9 ± 246.3	318.6 ± 52.8	568.1 ± 206.7
Benzyl nitrile	23.5 ± 1.9	64.9 ± 17.4	88.9 ± 23.9	131.2 ± 8.8	176.6 ± 9.9	218.4 ± 18.8	45.2 ± 5.2	52.6 ± 8.3	59.3 ± 9.4	86.7 ± 8.1	68.5 ± 3.8	79.7 ± 7.3
E-α-Farnesene	47.4 ± 4.0	196.9 ± 27.9	161.2 ± 21.8	210.3 ± 15.1	387.8 ± 24.5	340.8 ± 22.2	74.3 ± 10.4	94.3 ± 11.5	88.7 ± 7.8	82.0 ± 4.3	69.3 ± 4.3	91.0 ± 12.3
Flower VOC [pg/flower*l]												
Benzaldehyde	336.0 ± 18.9	427.8 ± 27.9	416.8 ± 27.0	1522.3 ± 64.5	1540.8 ± 60.5	1785.6 ± 98.4	855.9 ± 44.4	805.0 ± 45.8	848.7 ± 53.1	1116.6 ± 35.3	1009.7 ± 30.1	1046.6 ± 28.0
1,3,5-Trimethylbenzene	25.8 ± 1.3	34.7 ± 2.6	31.0 ± 1.8	127.5 ± 5.4	129.6 ± 5.6	147.3 ± 7.8	65.2 ± 3.6	62.1 ± 3.8	69.1 ± 4.1	72.4 ± 1.9	65.9 ± 1.8	69.1 ± 1.9
Phenylacetaldehyde	226.2 ± 18.1	170.2 ± 17.7	180.0 ± 14.8	183.7 ± 16.8	176.8 ± 13.1	161.2 ± 13.1	347.0 ± 47.8	254.6 ± 23.0	304.9 ± 41.5	266.1 ± 26.7	243.5 ± 16.8	231.4 ± 21.6
Methyl benzoate	98.4 ± 4.7	82.2 ± 4.4	82.4 ± 3.9	118.2 ± 6.5	107.1 ± 5.2	98.8 ± 5.3	84.6 ± 5.8	88.4 ± 5.5	90.4 ± 5.9	111.3 ± 8.3	98.1 ± 6.0	83.4 ± 5.6
Benzyl nitrile	144.9 ± 10.9	103.4 ± 7.1	108.8 ± 8.7	92.6 ± 8.5	76.3 ± 6.2	57.1 ± 4.9	62.3 ± 7.2	73.0 ± 7.9	59.2 ± 5.6	128.7 ± 12.0	143.3 ± 9.4	110.7 ± 7.9
Methyl salicylate	8.8 ± 0.4	6.6 ± 0.4	6.3 ± 0.3	8.9 ± 0.7	7.3 ± 0.5	6.9 ± 0.5	9.4 ± 0.8	11.8 ± 1.1	10.6 ± 0.8	13.6 ± 0.9	9.7 ± 0.7	8.1 ± 0.5
Z-α-Farnesene	45.2 ± 2.4	38.8 ± 1.9	37.8 ± 2.0	125.0 ± 6.3	122.0 ± 6.6	126.6 ± 7.4	102.0 ± 7.7	97.8 ± 6.3	97.7 ± 7.1	58.3 ± 3.4	50.2 ± 2.4	49.3 ± 2.3
E-α-Farnesene	791.0 ± 40.9	586.9 ± 33.2	622.2 ± 33.0	813.5 ± 62.0	717.9 ± 47.7	580.2 ± 38.1	978.8 ± 74.4	853.9 ± 55.3	962.3 ± 68.5	1042.0 ± 68.9	879.8 ± 57.5	832.7 ±54.1

Table S2 | LDA loading values Loading values of the first and second linear discriminant function (DF1 and DF2) separating plant generations and treatment groups. DF1 explains 44.0%, and DF2 explains 23.3% of the variance between groups. Total petal area was excluded from the analysis due to collinearity with petal area. Also, leaf VOCs could not be included as they were not available for all plants

	Loading values of DF1	Loading values of DF2
Morphological traits		
Plant height [cm]	-0.076079773	-0.175217817
Flower number	0.008781509	-0.092859298
Bud number	0.423497181	0.273369065
Leaf number	0.176404615	0.229943638
Nectar [nl/3 flowers]	-0.145747262	0.637136433
Petal area/flower [mm^2]	-1.28181005	-0.09198888
Total petal area [mm^2]	n.a.	n.a.
Reproductive traits		
Silique number	-0.04760028	-0.295756535
Seed weight [mg/10 seeds]	0.060193161	0.33116108
Seed number	0.154670424	0.046064437
Seed viability [%]	0.04704356	0.033367515
Leaf VOC [pg/ l]		
1-Butene-4-isothiocyanate	n.a.	n.a.
Benzyl nitrile	n.a.	n.a.
E-α-Farnesene	n.a.	n.a.
Flower VOC [pg/flower*l]		
Benzaldehyde	-0.059678475	-0.40739848
1,3,5-Trimethylbenzene	0.140416673	0.206667465
Phenylacetaldehyde	0.08577066	-0.253077722
Methyl benzoate	-0.040703581	-0.028593748
Benzyl nitrile	0.083348219	0.001104192
Methyl salicylate	-0.087293613	0.659169218
Z-α-Farnesene	-0.036769696	-0.11862906
E-α-Farnesene	0.08250177	0.215556585

Chapter III Emergence of a floral colour polymorphism by pollinator-mediated overdominance

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Abstract

Maintenance of polymorphisms by overdominant selection is a fundamental biological concept with a firm place in evolutionary textbooks, but with very few unambiguous examples in natural populations. We have applied an eco-evo-devo approach to assess the selective forces maintaining a polymorphism with three floral morphs in the Alpine orchid *Gymnadenia rhellicani.* Field observations revealed that bee and fly pollinators exert opposite directional selection on flower colour, together maximising seed set in the intermediate morph. A combination of phenotypic, metabolomic, and transcriptomic analyses further showed that the morphs differ solely in the concentration of cyanidin pigments, which is linked to differential expression of an *anthocyanidin synthase* (*ANS*) gene. Finally, transcriptome-wide association mapping identified a SNP heterozygous in the intermediate morph, inserting a premature stop codon in an *ANS* regulating *R2R3-MYB* transcription factor. Altogether, these findings support the classic idea that polymorphisms can evolve by overdominant selection of a single locus.

Introduction

Overdominance is defined as fitness advantage of individuals based on heterozygosity at a single locus (Charlesworth & Willis, 2009). Historically, overdominance had been considered an important mechanism maintaining polymorphisms in natural populations, as it provides a simple explanation for the retention of multiple alleles in a community (Dobzhansky, 1955; Delph & Kelly, 2014). Although a range of putatively overdominant cases has been identified, very few are documented well enough to (1) establish a clear connection between genotype, phenotype, and the selective force, and (2) exclude any co-influence of other genetic loci, phenotypic traits, or selection pressures (Gemmell & Slate, 2006; Futuyma, 2009). Among the well-understood examples, most are associated with intense, short-term selective pressures such as host-pathogen interactions (Frelinger, 1972; Carrington *et al.*, 1999; Aidoo *et al.*, 2002) or artificial selection (Greaves *et al.*, 1977; Adalsteinsson, 1980; Fujii & Otsu, 1991; Gemmell & Slate, 2006; Hillbertz *et al.*, 2007; Karlsson *et al.*, 2007; Mosher *et al.*, 2007; Sellis *et al.*, 2016). In these cases, homozygosity of one allele often has a negative fitness effect, and cessation of selection would revert the fitness advantage of the heterozygote. Overdominance is thus often seen as a rather short-lived phenomenon, and its perceived significance in maintenance of polymorphisms has somewhat diminished in favour of other modes of selection (Gray & McKinnon, 2007; Hedrick, 2012).

Polymorphisms of visual traits such as colour have been studied for decades, as they can provide insights into fundamental evolutionary processes (Svensson, 2017). In plants, polymorphisms of floral organs are often highly

fitness-relevant due to their impact on pollinator interactions (Kay, 1978; Russell *et al.*, 2017). Most floral polymorphisms have been associated with antagonistic pleiotropy (Irwin *et al.*, 2003; Jersáková *et al.*, 2006; Carlson & Holsinger, 2010), negative frequency-dependence (M Eckhart *et al.*, 2006; Barrett, 2013), or spatiotemporal heterogeneity (Schemske & Bierzychudek, 2007; de Jager & Ellis, 2013; Pope *et al.*, 2014; Ortiz *et al.*, 2015). To our knowledge, the action of heterozygote advantage has been proposed in the colour polymorphic annuals *Cosmos bipinnatus* (Malerba & Nattero, 2012), *Ipomoea purpurea* (Subramaniam & Rausher, 2000), and *Sisyrinchium sp*. (Takahashi *et al.*, 2015). Selection in *Cosmos* may not be strictly overdominant as the colour morphs also differ in number and size of flowers (Malerba & Nattero, 2012); likewise, pollinator-mediated selection in the other two species seems to be co-influenced by other factors (pleiotropic effects of the colour locus in *Ipomoea* (Mojonnier & Rausher, 1997; Coberly & Rausher, 2008), and reproductive interference in *Sisyrinchium* (Takahashi *et al.*, 2016)).

A yet unexplored floral colour polymorphism exists in a population of the Alpine orchid *Gymnadenia* (*Nigritella*) *rhellicani* located on Alm Puflatsch (Seiser Alm) in Northern Italy (Table S1): Unlike in other locations, where flowers of *G. rhellicani* are uniformly black, strikingly, this population is highly polymorphic with 62% (wild-type) black, 28% red, and 10% white individuals (census 2015, Fig. 1a). Within the population, morphs are randomly distributed, often in close proximity (5 cm) to each other, and individuals do not seem to switch colour throughout their perennial life cycle. Variation in floral coloration is thus likely genetically determined. To our knowledge, this population cannot be older than ca. 800 years, when forests on Puflatsch were cleared (Göpfert, 2017); the first notion of colour polymorphism is from 1906 (Torre *et al.*, 1906), with a photographic record starting in 1971 (Reinhard, 1971). Moreover, frequency estimations conducted between 1997 and 2016 indicate an increase in relative abundance of the red and white morph from total <5% to >40% (Fig. 1b). This suggests the current action of strong selection pressure favouring the white, the intermediate, or both morphs in this system.

Results and discussion

To assess the selective forces maintaining this polymorphism, we first quantified both inflorescence survival (fraction of inflorescences eaten by insects and livestock as well as reappearance of inflorescences in the next season) and reproductive fitness (number of fruits × percentage of viable seeds) of 281 plants (Table S2). While there was no difference in inflorescence survival between morphs (Fig. S1a), red plants had a significantly higher seed set than either of the extreme morphs (Fig. 1c). This difference was mainly caused by variation in number of fruits, which develop only after successful pollination(Zhang & O'Neill, 1993), supporting the hypothesis of pollinator-mediated heterozygote advantage. Also, flow cytometry of collected pollinia identified all morphs as diploid (Fig. S1b), and shielding plants from pollinators with glass fibre mesh resulted in poor seed set (Fig. 1d), ruling out apomictic or autogamous reproduction as known from other polyploid *Gymnadenia* species (Hedrén *et al.*, 2000).

G. rhellicani is a nectar-producing plant visited by > 60 different insect species from various orders (Müller, 1874; Vöth, 2000), and observations at Puflatsch have shown that pollination takes place during daytime with bees and flies as main carriers of pollinia (R.T.K. and K.J.R.P.B., pers. obs.). To test for differences in pollinator visitation, we caught / surveyed floral visitors (Table S3) and placed ten cameras in front of (1) *in situ* pairs of black and red plants for four consecutive days in 2016, and (2) triplets of freshly cut inflorescences (one per morph) for three days in 2017, taking pictures every 60 s (2016: 25007 frames, 2017: 14419 frames). In 2016, we counted 160 landings of insects (18% bees, 81% flies, and 1% other), and in 2017, we recorded 197 landings (24% bees, 64% flies, and 12% other). While bees, which are less common but likely more efficient pollinators, visit more dark than bright morphs, flies on the contrary visit more bright than dark morphs (Fig. 1e, Fig. S1c). Different preferences by bees and flies thus seem to exert opposite directional selection pressures on flower colour, providing an explanation for the observed fitness advantage of the intermediate colour morph. Mapping of spectral reflectance data (λ = 300 to 700 nm) of flowers (Fig. S1d) in the fly (Troje, 1993; Lunau, 2014) and bee (Chittka, 1992) visual spaces further suggested that both pollinator groups are able to distinguish the morphs based on luminosity (Fig. S1e and S1f).

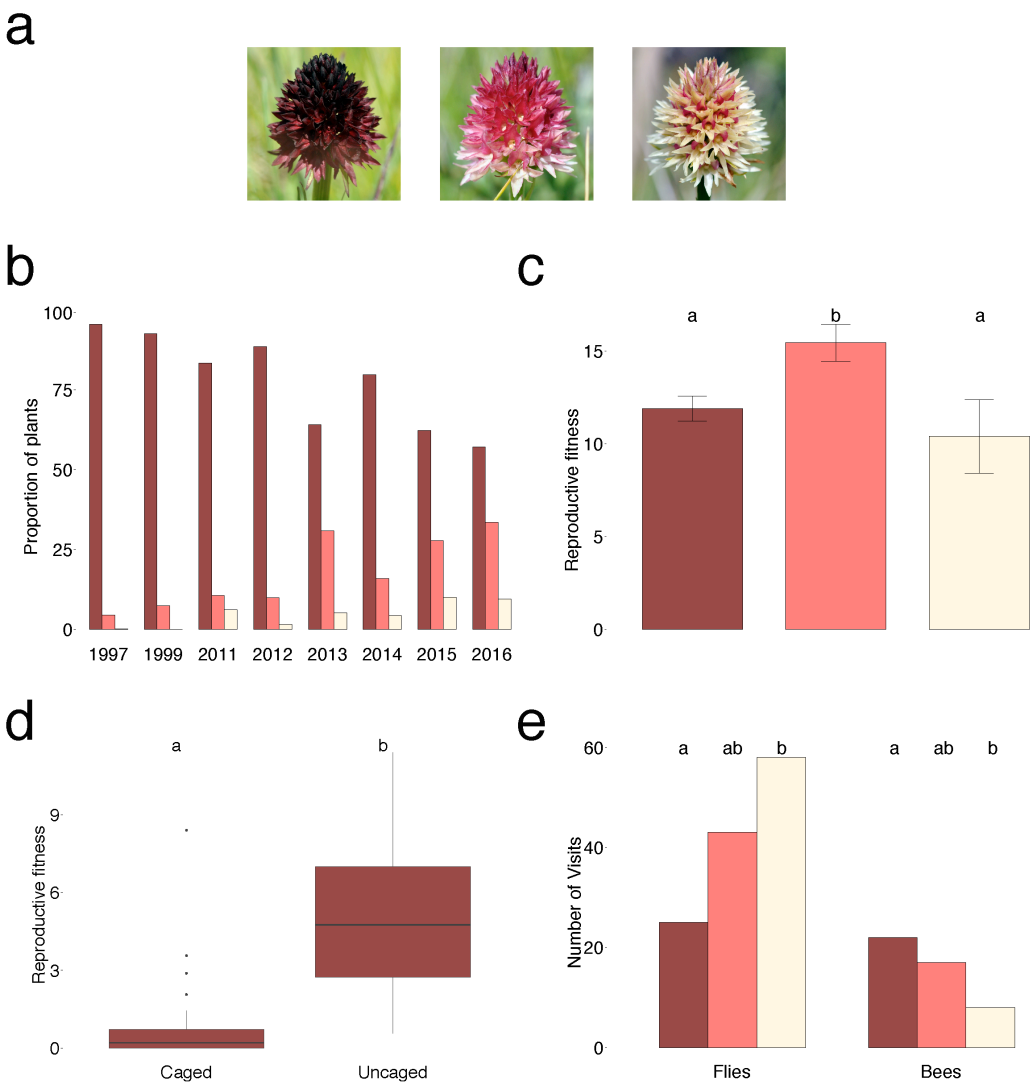

Figure 1 | Ecological evidence for overdominance in *Gymnadenia rhellicani* **a**, The *G. rhellicani* population on Alm Puflatsch (Seiser Alm) in Northern Italy contains three distinct floral colour morphs, which grow completely intermixed and in close proximity to each other. **b,** Frequency estimations indicate that the fraction of red morphs is increasing at Puflatsch (1997 - 2014 data: estimates, courtesy Richard Lorenz). **c,** As expected under overdominance, red, intermediate plants have a higher reproductive fitness (mean ± 1se of number of capsules produced after pollination × proportion of viable seeds) than both extreme morphs; ANOVA (n=178), $F(4, 168)=8.935$, $P=1.446 \times 10^{-6}$, and TukeyHSD *post hoc* test, $t_{Black\text{-}Red}=3.334$, $P_{Black\text{-}Red}=0.003$, $t_{Black\text{-}White}=-1.168$, $P_{Black\text{-}White}=0.467$, $t_{Red\text{-}White}=-3.250$, $P_{Red\text{-}White}=0.004$. **d**, Plants at the ungrazed Ofenpass site shielded from pollinators with glass fiber mesh resulted in poor seed set, indicating that reproduction in *G. rhellicani* is pollinator dependent (t test (n=42), $t(34.818)=-4.939$, $P=1.955 \times 10^{-5}$, the high outlier value was caused by an accidentally trapped grasshopper leaving a hole in one of the nets). **e,** Time lapse video recordings from 2017 show that bees preferentially visit dark plants, while flies prefer bright plants, resulting in opposite directional selection favouring the red morph (χ^2-test bees (n=47); $\chi^2=6.426$, $P=0.040$, χ^2-test flies (n=126), $\chi^2=13.000$, $P=0.002$, χ^2-test others (n=24), $\chi^2=0.750$, $P=0.687$).

Considering these findings, bees and flies could either distinguish morphs by flower colour only, or based on pleiotropic effects of other phenotypic traits. We therefore quantified a series of other potentially pollinator-relevant floral traits including plant height, flower number, and inflorescence temperature in the morning and at noon, and performed 3D-morphometric scans to assess floral morphology. Our analyses showed that the three colour morphs do not differ in any of these traits (Fig. S2a-d). We also collected floral volatiles by headspace sorption and gas chromatography mass-selective detection (GC-MSD) and assessed the composition of the floral volatile bouquet (Tava *et al.*, 2012). Both the whole volatile bouquet, and the emission of individual scent compounds were not different between the colour morphs (except for one minor compound, Fig. S2e, Fig. S3, Table S4). A temporal comparison of volatile emission at 15:30 and 21:00 further showed that all morphs reduce volatile emission after sunset (Fig. S2f). Altogether, these results suggest that the underlying mutation(s) only affect flower coloration, which may thus be under overdominant selection.

To characterise the molecular basis of this polymorphism, we combined the phenotypic measurements with metabolomic and transcriptomic data from the same individuals: We extracted anthocyanin and carotenoid pigments as well as RNA from the same pool of four open flowers per individual. Quantification of anthocyanins and their colourless precursors with ultra high-performance liquid chromatography (UHPLC-MSMS) identified cyanidin-3-glucoside and a derivative, putatively cyanidin-3-(6-malonylglucoside), as dominant pigments in the flowers. Another derivative, peonidin-3-glucoside, was also present to a lesser degree. The abundance of the two main cyanidin compounds is more than 7.5× lower in red, and more than 30× lower in white plants than in the black morph (Fig. 2b, Fig. S4, Tables S5 & S6). In contrast, UHPLC-MSMS quantification of carotenoids showed that the flowers mainly contain β-carotene and lutein with no difference in concentration between the three morphs (Fig. S2g). Next, we performed mRNA-seq of one plant per morph, three other black *G. rhellicani* plants from other populations, and four individuals of three closely related *Gymnadenia* species. We *de-novo* assembled the Illumina HiSeq 2500 reads to a combined reference transcriptome, yielding a total of 836101 contigs with a N_{50} length of 553 bp. With the remaining samples, we prepared multiplexed libraries for RNA expression profiling by low-coverage mRNA-seq (Illumina HiSeq 2500), resulting in 166 million reads that were mapped to the reference transcriptome. We next performed differential expression analysis between all three morphs and found nine transcripts with a significantly different expression level. The top differentially expressed transcript could be assigned to an *anthocyanidin synthase* (*GrANS1*) gene, a key member of the anthocyanin pathway (Fig.2a & 2c, Fig. S4, Table S7).

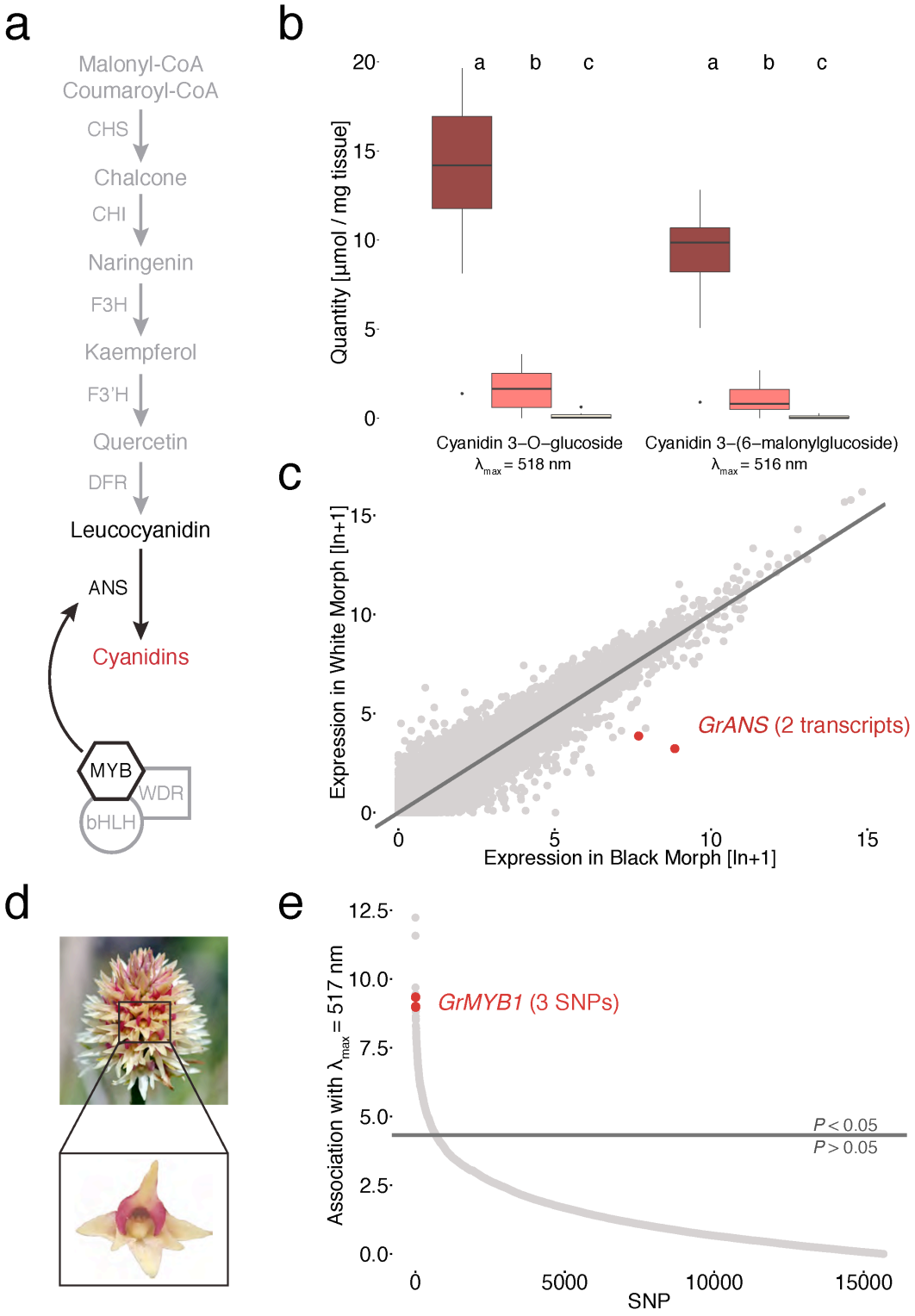

Figure 2 | Molecular-developmental basis of the colour polymorphism a, Simplified diagram of the cyanidin branch of the anthocyanin pathway (the delphinidin and pelargonidin branches play no role in *G. rhellicani,* Fig S4). The last step from colourless leucocyanidin to coloured cyanidins is mediated by an *anthocyanidin synthase* (*ANS*) typically regulated by *MYB-bHLH-WDR* transcription factor complexes. **b,** Concentrations of the two main anthocyanin pigments in *G. rhellicani* flowers, cyanidin-3-glucoside and its derivative, putatively cyanidin-3-(6-malonylglucoside), are reduced > 7.5 × in red,

and > 30 × in white plants; c.-3-O-g.: ANOVA (n=47), $F(2, 44)=97.900$, $P<2.2 \times 10^{-16}$, and TukeyHSD *post hoc* test, $t_{Black\text{-}Red}=-7.387$, $P_{Black\text{-}Red}<1 \times 10^{-7}$, $t_{Black\text{-}White}=-13.981$, $P_{Black\text{-}White}<1 \times 10^{-7}$, $t_{Red\text{-}White}=-6.366$, $P_{Red\text{-}White}=1.55 \times 10^{-7}$, c.-3-6-mg.: ANOVA (n=47), $F(2, 44)=98.300$, $P<2.2 \times 10^{-16}$, and TukeyHSD *post hoc* test, $t_{Black\text{-}Red}=-7.422$, $P_{Black\text{-}Red}<1 \times 10^{-6}$, $t_{Black\text{-}White}=-14.008$, $P_{Black\text{-}White}<1 \times 10^{-6}$, $t_{Red\text{-}White}=-6.358$, $P_{Red\text{-}White}=1 \times 10^{-6}$. **c,** Differential expression analysis between black and white colour morphs: The most differentially expressed transcripts map to *ANS* (Table S7). **d,** Close-up of a flower from a white morph; the lateral lobes of the lip still contain anthocyanin pigment, suggesting a mutation in a regulatory pathway element. **e,** Transcriptome-wide association between SNPs called from the expression profiling data (3'-ends of transcripts) and the spectral reflectance at the absorption maximum of the two main cyanidins (517 nm): Three of the ten SNPs with strongest association (position 7, 8, and 9) occur in the same *MYB* transcription factor (*GrMYB1,* Table S8).

A closer inspection of flowers from white morphs shows that flowers are not uniformly white but that the lateral lobes contain residual anthocyanin (Fig. 2d), suggesting a mutation in a regulatory element rather than in the *GrANS1* gene itself. To find the underlying mutation, we identified SNPs from the expression profile data and performed a transcriptome-wide association study (TWAS). Here, spectral reflectance at 517 nm wavelength was used as phenotype, since this is the mean of the two main cyanidins' absorption maxima (516 and 518 nm). Three of the top ten associated SNPs mapped to the 3' non-coding region of the same *GrMYB1* gene, belonging to a family of *R2R3-MYB* transcription factors involved in the regulation of anthocyanin production (Fig. 2a & 2e, Table S8). Since the expression profiles cover only the 3'-end of transcripts, these SNPs may not be causative themselves, but indicate the presence of alleles segregating according to colour morph. Closer inspection of the full *GrMYB1* transcript revealed a non-synonymous SNP with three allelic states C (ancestral 'wild-type'), G, and A in the last exon of the coding region (Fig. 3). Genotyping of all plants showed that black plants with high cyanidin content and *GrANS1* expression are homozygous for the wild-type allele (C/C), white plants with low cyanidin content and *GrANS1* expression never contain the wild-type allele (G/G, G/A, or A/A), and the red, intermediate morph is heterozygous for the wild-type allele (C/G or C/A). Both transversions (C→G, and C→A) introduce a premature stop codon, consistent with a loss of function of the derived alleles by truncating the protein by 43 amino acids. The observed segregation pattern is thus fully consistent with overdominance.

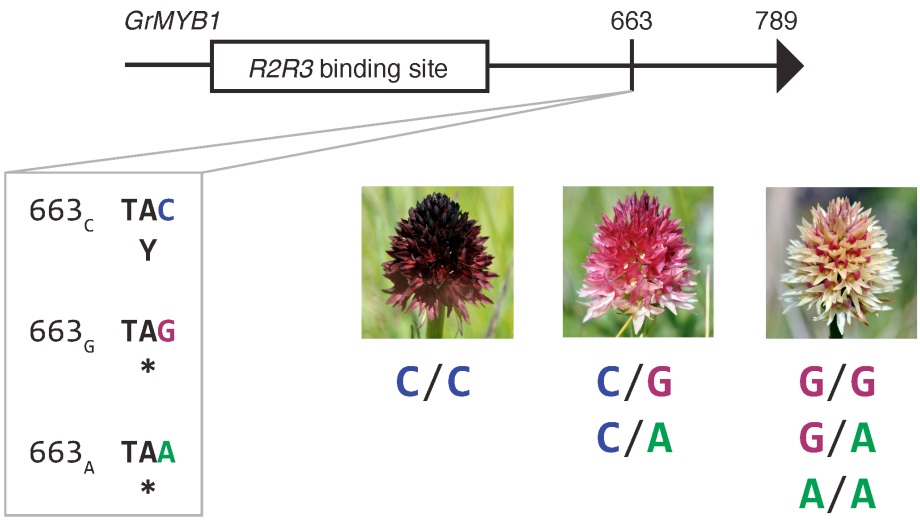

Figure 3 | Genetic evidence for overdominance in *Gymnadenia rhellicani* The polymorphic *G. rhellicani* population located on Alm Puflatsch contains a single nucleotide polymorphism (SNP) with three variants at position 663 in the last exon of the *GrMYB1* coding sequence. While the ancestral variant 663_C codes for Tyr (Y), both 663_G and 663_A code for a stop codon and expected to truncate the protein by 43 amino acids. As expected under overdominance, black plants are always homozygote for 663_C, red plants are always heterozygote for 663_C, and white plants never contain 663_C.

In several cases, follow-up studies on putative overdominant systems have shown that heterozygosity is not favoured due to a direct fitness effect of the identified locus, but rather due to pleiotropic loci under selection (associative overdominance)(Frydenberg, 1963; Zhao & Charlesworth, 2016). Although direct functional verification of an interaction of *GrMYB1* with the differentially expressed *GrANS1* gene is currently infeasible in *G. rhellicani*, we conducted follow-up investigations which strongly indicate a direct effect of *GrMYB1* on plant colour patterning: First, we reconstructed the phylogenetic relationship of *GrMYB1* to other *MYB* gene copies from *Arabidopsis thaliana* and the orchid *Phalaenopsis.* Our analysis places *GrMYB1* within a clade consisting of *A. thaliana MYB* subgroup 6, and *Phalaenopsis equestris MYB 2, 11,* and *12,* all of which have been shown to (co)regulate *ANS* expression patterning in these plants(Gonzalez *et al.*, 2008; Hsu *et al.*, 2015) (Fig. 4a). Second, we applied double-stranded RNA to cut inflorescences of wild-type *G. rhellicani* plants from a natural population so as to reduce *GrMYB1* expression by RNA interference. Of twenty-two treated plants (many of which were already at an advanced stage of flowering), one showed a weak, and one a clear reduction in coloration of newly opening flowers (Fig. 4b), the expression of both *GrMYB1* and *GrANS1* being significantly decreased in the latter plant (Table S9). Third, we analysed the coding sequence of *GrMYB1* in 98 individuals from 14 other *G. rhellicani* populations distributed across the Alps (Table S1). Most of these populations contain wild-type black plants with very rare occurrences of uniformly red or yellow flowers (<0.1%

mutants), all of which contain the wild-type *GrMYB1* alleles (Fig. 4e). However, one smaller population located ca. 75 km south-west of Puflatsch at Monte Bondone, Italy (Table S1), contains ca. 20% plants with a phenotype resembling the red, and <1% plants with a phenotype resembling the white morph at Puflatsch (census 2015). Genotyping revealed that the nucleotide at an ancestral SNP position (R = G or A, encoding Met or Ile) in the last coding exon of *GrMYB1* is deleted in alleles of all red and white plants analysed (Fig. 4c). Not only the segregation pattern of this deletion (black: R/R, red: R/-, white: -/-), but also its predicted effect on the protein (frameshift and truncation of 54 amino acids) are completely analogous to the Puflatsch population. Nevertheless, the mutation discovered in the Puflatsch population does not occur at Bondone and vice-versa, implying that both mutations must have evolved independently (Fig. 4d & 4e).

Taken together, our data suggest that floral colour polymorphisms can evolve by pollinator-mediated overdominant selection. In addition, the two investigated polymorphic populations likely represent a previously unknown case of parallel evolution.

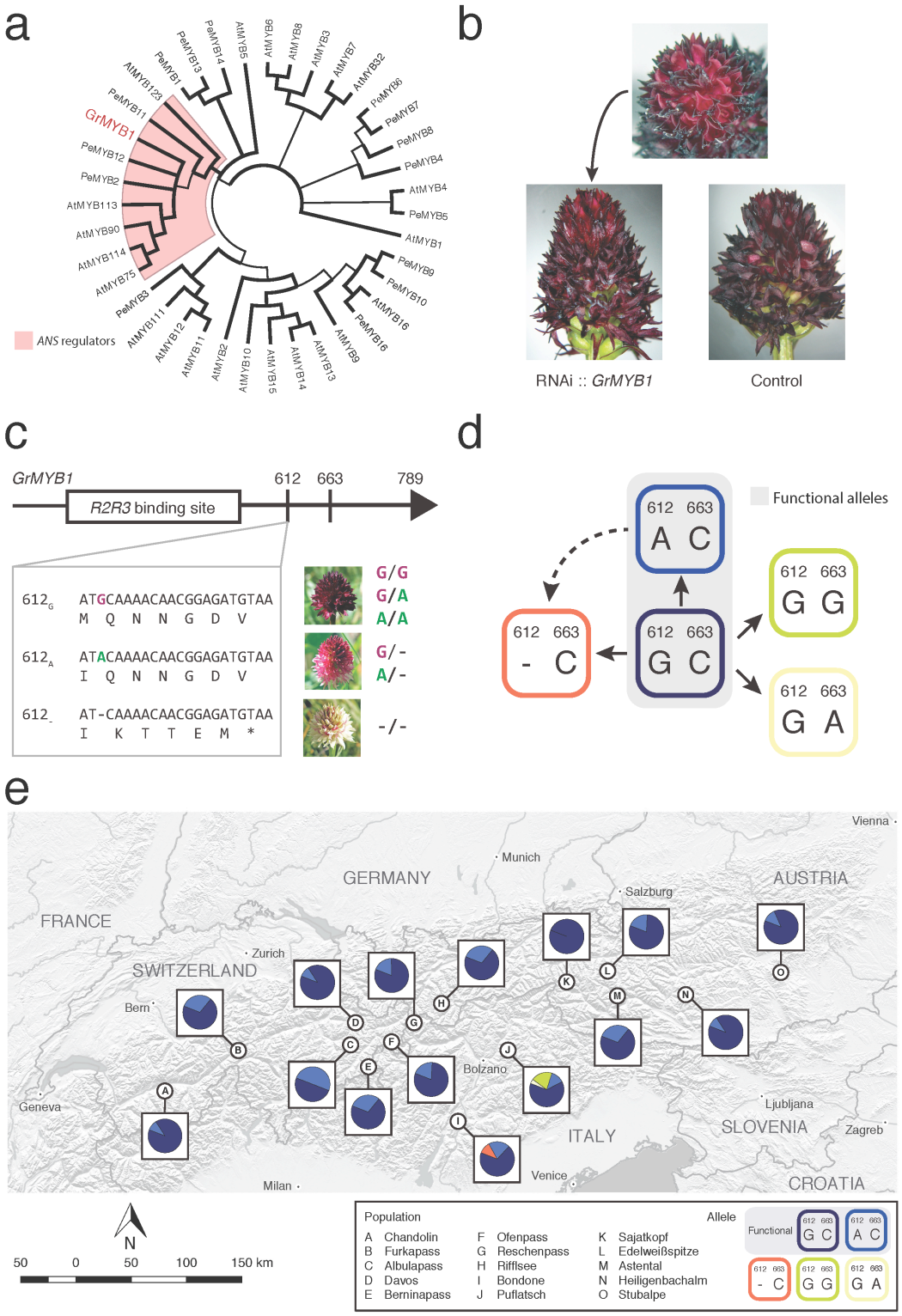

Figure 4 | Indications of a functional role of *GrMYB1* in flower coloration a, Bayesian phylogenetic analysis of the conserved *R2R3-MYB* binding domain from *Arabidopsis thaliana* (*AtMYB*) and *Phalaenopsis equestris* (*PeMYB*) places *GrMYB1* in the clade of *AtMYB* subgroup 6, and *PeMYB2, 11,* and *12*, all of which (co)regulate *ANS* expression. Branches with posterior probability > 80% are in bold. **b,** RNA interference (RNAi) with the last coding exon of *GrMYB1* results in partial pigment loss in developing flowers, and in reduced expression of *GrMYB1* and *GrANS1* (Table S9). **c,** Analogous to the Puflatsch population (Fig. 3), *G. rhellicani* plants at Bondone contain a SNP with three variants at

position 612 in the *GrMYB1* coding sequence. Black plants contain 612_G and/or 612_A, coding for Met (M) and Ile (I), a polymorphism also found in wild-type populations (Fig. 4e). Red and white plants, however, are hetero-, and homozygous for a deletion of this nucleotide, resulting in a frame shift and the expected loss of 54 amino acids. Selection at Bondone is yet unstudied, but this pattern also fits the overdominance hypothesis. **d,** Evolutionary model of *GrMYB1*-alleles in *G. rhellicani* (Fig. 4e). Both the blue and the light blue allele are functional; the blue allele is likely ancestral as it is the commonest across most populations. Plants homozygous for 633_G and 633_A never contain 612_A, and two further SNPs at position 849 and 864 in the 3'-UTR of 633_C co-segregate with 633_G and 633_A. This indicates that the green and yellow alleles must have evolved independently from the blue allele. Since 612_- is always associated with 633_C, the red allele must have evolved independently from green and yellow, either from the blue, or less likely, from the light blue allele. **e,** Relative *GrMYB1* allele frequencies in 15 *G. rhellicani* populations across its Alpine habitat: The functional blue and light blue alleles co-occur in almost all populations, the green and yellow alleles only occur at Puflatsch, and the red allele only occurs at Bondone. Map based on ALOS World 3D - 30m digital surface model (© JAXA) and administrative boundaries from gadm.org.

Material and Methods

For detailed methods see supplementary methods, for detailed sample sizes see Table S2. To enable cross-comparisons, analyses included the same set of 48 plants distributed over both ridges of the Puflatsch population whenever possible (see below and Table S2).

Study site

The main polymorphic *G. rhellicani* population contains around 4500 individuals distributed mainly over two ridges in east-west extension on the volcanic outcrop of the Alm Puflatsch, Italy (Table S1). The whole area is subject to intense browsing by cattle and horses during summer. Also, lower parts of both ridges are usually mown in early autumn. To keep track of individual plants over multiple seasons, they were invisibly marked by burying passive integrated transponder (PIT) tags (HPT23, Biomark, Idaho) in 1.5 ml test tubes close to the shoots. Re-localisation of tagged plants was done with a handheld GPS receiver (Garmin, Switzerland) and a Biomark HPR Plus reader with a BP Plus portable antenna. The second polymorphic *G. rhellicani* population contains around 650 individuals and is situated on Monte Bondone, Italy (Table S1). For the locations of all other wild-type populations see Table S1.

Fitness measurements

Fitness quantification of the colour morphs was based on fourteen 2 × 2 m plots containing 281 plants in total (Table S2). To assess plant survival, the number of damaged and eaten plants as well as the number of plants, which did not reappear in the consecutive flowering season, was recorded for each colour morph. To assess plant reproduction, the number of capsules per plant was determined from close-up photographs, and seed viability was estimated by determining the presence / absence of embryos under a microscope.

Pollinator experiments

To test whether seed set in *G. rhellicani* is pollinator-dependent, black wild-type plants in a non-browsed population at Ofenpass, Switzerland (Table S1) were covered in bud stage with glass-fibre nets, and capsule number and seed quality were recorded as described above. Preferences of pollinators for colour morphs were recorded by placing time-lapse cameras (1 frame/min) in front of an *in-situ* pair of a red and black morph in 2016, and in front of three cut inflorescences (one per morph) in 2017.

Phenotypic analyses

To assess the ploidy of the studied plant individuals, pollinia were collected and analysed with flow cytometry. For determining insect perception of the colour morphs, spectral reflectance measurements of flowers were taken in the UV and visible wavelength range (300-700 nm), and the obtained spectral curves were mapped in the bee and fly visual space. Plant height was recorded for 781 plants, and inflorescence temperatures were determined with an infrared thermometer in the morning and afternoon of a cloudless day. Three-dimensional flower morphology was obtained by micro-computed tomography (µCT) and quantified by placing landmarks on the petals, lip, column, and spur of scanned flowers. Floral volatile organic compounds (VOCs) were collected with headspace sorption and analysed using gas chromatography with mass selective detection (GC-MSD). To assess daily fluctuations in VOC emission, VOCs were collected from the same plants in the morning, afternoon, and at night of a cloudless day.

Metabolomic analyses

Anthocyanins were extracted from flowers at the top, centre, and bottom part of the inflorescences and separated with ultra-high performance liquid chromatography (UHPLC) coupled with Compact Electrospray Ionization-Quadrupole-Time-of-Flight (ESI-Q-TOF). Individual compounds were identified and annotated by comparison with authentic reference standards data, except for cyanidin-3-(6-malonylglucoside), which was tentatively identified based on the data spectrum obtained and previous results. Peak areas of the identified compounds were standardised by flower tissue weight and the molecular weight of the compound. Concentrations of the two main compounds cyanidin-3-glucoside and cyanidin-3-(6-malonylglucoside) were calculated based on a calibration curve of cyanidin-3-glucoside. Carotenoids were re-extracted from the anthocyanin samples and subjected to saponification prior to high performance liquid chromatography (HPLC) analysis. The two main carotenoids β-carotene and lycopene were identified by comparison with authentic reference standards data and quantified as described above.

Transcriptomic analyses

A *de-novo Gymnadenia* reference transcriptome was constructed by performing mRNA-seq on an Illumina HiSeq 2500 platform (Illumina, California) for one plant per morph from the Puflatsch focal plants, one plant from Ofenpass, two plants from Chandolin (Table S1), and one plant each of three other *Gymnadenia* species (*G. densiflora, G. odoratissima,* and *G. conopsea*) from other Swiss localities. For differential expression analysis, expression profiles were generated on an Illumina HiSeq 2500 platform using a Lexogen QuantSeq 3' mRNA-Seq kit (Lexogen, Austria). Expression profile reads were trimmed, mapped to the reference transcriptome, and posterior mean counts were determined for each morph. Transcripts with a significant expression difference were searched against the NCBI nucleotide database. For transcriptome-wide association study (TWAS), variant calling was performed for the mapped and indexed expression profile reads, and the association of variants with the normalised spectral reflectance at 517 nm wavelength (the mean absorption maximum of the two main anthocyanins) was assessed. Sequences of the ten transcripts with the strongest association were searched against the NCBI nucleotide database.

Genetic analyses

For phylogenetic analysis, sequences of the conserved *R2R3* domain were extracted from *Arabidopsis thaliana* and *Phalaenopsis MYB* genes, aligned with *GrMYB1,* and used for bayesian phylogenetic analysis. For genotyping, RNA or DNA was extracted from plants at Puflatsch, Bondone, and all other thirteen populations (Tables S1 and S2). Full or partial *GrMYB1* coding sequence was amplified and Sanger-sequenced. For RNA-interference (RNAi), full coding sequence of the last exon of the wild-type *GrMYB1* allele was amplified and cloned into a *p*L4440 destination vector, a gift from Andrew Fire (Addgene plasmid # 1654). The *E. coli* strain HT115 (courtesy Anita Dirks) was transformed with this construct for dsRNA-production. Crude bacterial lysate was extracted and applied to whole, expanding *G. rhellicani* inflorescences cut at Ofenpass using carborundum. After 7 days, five flowers each from two treated plants showing a phenotype and four mock-inoculated control plants were collected, and the expression levels of *GrMYB1* and *GrANS1* were determined with RT-qPCR.

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Supplementary Information

Supplementary Methods

Detailed sample sizes are listed in Table S2. To enable cross-comparisons, analyses included the same set of 48 plants distributed over both ridges of the Puflatsch population whenever possible (Table S2).

Fitness measurements

In July 2015, 14 plots were established in the Puflatsch population with a dimension of 2 × 2 m, containing a total of 281 plants (Table S2). The plots were evenly distributed in 10 m interval along the east-west axis of the entire Northern ridge, and a random azimuth of 0-5 m to the north or south. Within the plots, position, height, and colour of each plant was recorded, and all inflorescences were photographed. Number of flowers per inflorescence was inferred from these photographs by hand-counting individual flowers with the aid of a custom script implemented in *R* (*R* v.3.2.4, *R* Development Core Team 2016). To correct for the fraction of flowers invisible on the photo, flower numbers were multiplied with a factor of 1.22, which was determined by comparing flower numbers between photos and actual inflorescences of 30 individuals. In September 2015, the number of eaten plants was recorded, differences in herbivory between morphs were computed in *R* with a χ^2 test, and the remaining infructescences were photographed. Number of capsules was inferred using the aforementioned custom *R* script. To assess seed quality, the content of five capsules per individual was photographed under a microscope with transmitted light and 20 × magnification (Carl Zeiss, Germany). Since orchid seeds are transparent and do not contain an endosperm, seed viability can be estimated by looking at embryo presence. Viability was estimated from 100 seeds per plant as described for the flowers. Number of capsules was multiplied with relative seed viability, and fitness values > 0 were BoxCox transformed using the *R* package *caret* v. 6.0-76. Relative reproductive fitness differences between morphs were then computed in *R* applying a TukeyHSD *post-hoc* test on an AIC optimised linear model with colour, standardised plant height, and standardised flower number as fixed effects. In July 2016, the plots were revisited and differences in re-flowering between morphs were computed in *R* with a χ^2 test.

Pollinator exclusion

To avoid incidents with livestock, this experiment was conducted in a non-browsed wild-type population on top of Ofenpass, Switzerland (46°38'02"N 10°17'02"E, ca. 2290 m elevation, Table S1). Analysis of SNP data derived from low-coverage mRNA-seq (see below) with *Structure* v. 2.3.4 as described in (Sedeek *et al.*, 2014) showed that this population shares its genetic ancestry with

the colour-polymorphic population at Puflatsch. In June 2016, 40 plants in bud stage were randomly selected and PIT-tagged, and 20 plants were covered with white glass fibre nets (1 mm mesh size) attached to cylindrical metal frames of 25 cm height × 10 cm diameter. In September 2016, capsules were counted and seed quality as well as fitness differences were determined as described earlier.

Pollinator recordings

During peak flowering in July 2016, ten Somikon (Pearl, Switzerland) and one Brinno TLC 200 (Brinno, Taiwan) time-lapse camera were placed in front of *in situ* pairs of black and red plants growing in ca. 10 cm distance of each other. The plants were recorded in a 1 min interval during four consecutive days, with cameras shutting off automatically at night. Two people (R.T.K. and K.J.R.P.B.) independently screened all images for number and type of pollinators. In July 2017, pollinator recordings were repeated for three consecutive days: Three freshly cut inflorescences (one per morph, randomly chosen) were randomly placed in ca. 5 cm distance of each other in buried 15 ml tube filled with water in front of each camera (9 Somikon and 1 Brinno). Differences in visitation of pollinator groups between morphs were calculated in R with holm adjusted χ^2 tests.

Flow cytometry

Ploidy analysis was based on a protocol in (Gross & Schiestl, 2015) with minor modifications. Pollinia from three flowers per individual were ground in 200 µl Otto 1 buffer (0.1 M citric acid monohydrate in 0.5% Triton X-100) in a 1.5 ml reaction tube using a pistil. An internal standard solution was prepared by grinding 2 cm^2 of *Phaseolus coccineus* leaf in 1 ml Otto 1 solution, and filtering the suspension through a 30 µm CellTrics filter (Sysmex, Germany). After addition of 5 µl standard solution, each sample was filtered through a 30 µm CellTrics filter and centrifuged for 5 min at 380 × *g*. The supernatant was removed, and nuclei were resuspended in 40 µl Otto 1 buffer. Samples were analysed on a Beckman Coulter Cell Lab Quanta flow cytometer (Beckman Coulter, California) loaded with 160 µl Otto 2 solution (0.4 M disodium phosphate heptahydrate with 4 µg/ml 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) added before usage). Ploidy was determined as ratio between the medians of the standard and sample peaks in the flow cytometric histogram. As *G. rhellicani* is a diploid plant (Hedrén *et al.*, 2000), the lowest sample peak detected was assumed to represent the haploid pollinaria of diploid individuals, and the two adjacent peaks correspond to the diploid and tetraploid state.

Spectral mapping

Spectral measurements were taken with an AvaSpec spectrometer (Avantes, Netherlands) from three flowers per individual. The three spectral curves per sample were averaged between 300 and 700 nm wavelength, smoothed, and normalised. The curves were mapped into the fly visual space using a custom *R* script based on (Troje, 1993), and into the bee visual space using an *R* script published in (Sedeek *et al.*, 2014) with minor modifications.

Temperature measurements

Inflorescence temperature was measured in the morning between 9:00 and 10:00 (60 plants) and in the afternoon between 12:00 and 13:00 (90 plants) on a cloudless day in July 2016. Temperatures were recorded with an infrared thermometer (Model JHK-6606, Walter Werkzeuge, Austria) in two transects from east to west along the bottom and top part of the Northern ridge.

3D morphometrics

In July 2014, one flower from the lower part of the inflorescence was collected and stored in 1.5 ml FAA fixative (50% ethanol, 5% glacial acetic acid, 3.7% formaldehyde). Flowers were infiltrated with a contrasting solution (1% phosphotungstic acid in 70% ethanol) and subsequently embedded in acryl pillow foam (DACRON®COMFOREL®, Invista, USA) according to (Staedler *et al.*, 2013). Three-dimensional flower morphology was obtained by micro-computed tomography (µCT), using a MicroXCT-200 imaging system (Xradia, USA) with a 90kV Microfocus X-ray. All samples were scanned with the following parameters: acceleration voltage: 37kV; source current: 200µA; exposure time: 1,5s; number of exposures: 728; camera binning: 4; objective: LFOV (magnification = 0,4 ×); voxels size: 38,7475 µm. Three-dimensional data was exported in DICOM format. In *Amira* v. 5.3.3 (Zuse Institute, Germany), a total of 21 geometric landmarks were positioned on each scanned flower: one at the base and tip of each petal, labellum, and column, three along the left and right side of the labellum, one in the centre of the labellum, and one at the tip of the spur (Fig. S2d). Landmark positions were then compared among the three colour morphs with procrustes ANOVA from the *R* package *geomorph* v. 3.0.3 (Adams & Otárola‐Castillo, 2013).

Floral volatile analysis

Volatile organic compounds (VOC) were collected with non-destructive headspace sorption, a standard method also used in other *Gymnadenia* studies(Huber *et al.*, 2005): Entire inflorescences were enclosed in Toppits PET oven bags (Cofresco Frischhalteprodukte, Germany) and attached to a battery-driven pump connected to a glass tube loaded with 20 mg Tenax TA (60/80 mesh, Supelco, Pennsylvania). During 40 min, air was pulled from the bag through the Tenax filter at a flow rate of 100 ml min^{-1}. Background VOC levels

were determined with samples from empty oven bags. Analysis of VOC samples was done using gas chromatography with mass selective detection (GC-MSD) as described in (Gervasi & Schiestl, 2017). Identification and quantification of compounds was performed with the Agilent MSD ChemStation program E. 02.02 (2011) based on a mass spectral library built on calibration curves using three to five different concentrations of authentic reference standards. To assess possible temporal fluctuations in intensity and composition of the scent bouquet, VOCs of 14 additional plants on the Southern ridge were collected at three different time points (10:00, 15:30, and 21:00) in July 2015 and analysed as described above. Emission of the 15 compounds contibuting > 1% to the floral bouquet was converted to pg l^{-1} air and BoxCox transformed to approach normality using the *R* package *caret* v. 6.0-73 (Kuhn, 2015). Differences in VOC emission between colour morphs was compared with (1) principal component analysis, and (2) Holm-adjusted linear mixed-effects models with sampling year as random factors and Tukey HSD *post-hoc* tests (*R* packages *nlme* 3.1-131 (Pinheiro *et al.*, 2009) and *multcomp* 1.4-6 (Hothorn *et al.*, 2008)).

UHPLC-MS/MS of anthocyanins

In July 2014, four flowers per plant were collected from the top, centre, and bottom part of the inflorescence respectively. Flowers were flash-frozen in liquid N_2 and ground to powder using a tissue lyser (Retsch Technology, Germany). 50% of each sample were weighed, transferred to a 2 ml tube, and flavonoids were extracted in 500 µl of 80% methanol (MeOH). Samples were ground again in solution, incubated overnight at 4 °C, and centrifuged at 11000 × *g* and 4 °C for 10 min. Supernatants were transferred to 1.5 ml tubes, evaporated in a Savant SpeedVac concentrator (Thermo Fisher Scientific, Massachusetts) at 42°C, and resuspended in 100 µl 50% MeOH + 0.1% formic acid. After sonication for 5 min, samples were centrifuged at 11000 × *g* and 4 °C for 5 min, and transferred to liquid chromatography (LC) vials. Samples were run on a UHPLC (Thermo Scientific Dionex UltiMate 3000) coupled to a Bruker Compact Electrospray Ionization-Quadrupole-Time-of-Flight (ESI-Q-TOF, Bruker Daltonics). The UHPLC separation was performed with a C18 reverse-phase column (ACQUITY UPLC TM BEH C18, 1.7µm, 2.1 × 150mm; Waters) at 28°C using the following gradient of solvent B (acetonitrile with 0.1% [v/v] formic acid) and solvent A (water with 0.1% [v/v] formic acid): 0-0.5 min, 5% B; 0.5-12 min, 5-100% B; 12-14 min, 100% B; 14-16min, 100-5% B. The flow rate was set up to 0.3 mL min^{-1} and 5 µL of each sample was injected. The ESI source was operated in positive mode and parameters were set as follow: gas temperature, 220°C; drying gas: 9L min^{-1}; nebuliser: 2.2 Bar; capillary voltage: 4500V; end plate offset: 500V. The instrument was set to acquire an m/z range of 50-1300. Conditions for MS/MS were set as described by Christ *et al.* (2016)(Christ *et al.*, 2016). All data were recalibrated internally using pre-run injection of 10 mM sodium hydroxide in 0.2% formic acid, 49.8% water, 50% isopropanol [v/v/v]. Data Analysis (version

4.2, Bruker Daltonics) and TargetAnalysis (version 1.3, Bruker Daltonics) were used to analyse the data. Absolute flavonoid quantification was based on standard curves and this analysis was performed using QuantAnalysis software (version 2.2 Bruker Daltonics). Flavonoids were identified and annotated by comparison with authentic reference standards data: m/z and MS/MS data, UV spectrum and retention time profiling except for cyanidin-3-(6-malonylglucoside), which was tentatively identified by an interpretation of the data spectrum obtained and based on previous results (Schütz *et al.*, 2006). The identified peaks were integrated and standardised by flower tissue weight and the molecular weight of the compound. Concentrations of the two main compounds cyanidin-3-glucoside and cyanidin-3-(6-malonylglucoside) were calculated based on a calibration curve of cyanidin-3-glucoside. For comparison between morphs, standardised quantities were BoxCox transformed and assessed compound-wise with Holm-adjusted ANOVA and TukeyHSD *post hoc* tests using the *R* package *caret* v. 6.0-76 (Kuhn, 2015).

HPLC-MS/MS of carotenoids

Carotenoids were re-extracted from the anthocyanin samples (see above). 80 µl hexane were added to each sample, the solution was vortexed and incubated on ice for 10 min. After centrifugation for 2 min at 11000 × *g*, the upper phase was removed with a glass syringe and transferred to an amber glass vial (Supelco, Pennsylvania). Samples were evaporated in a Savant SpeedVac concentrator (Thermo Fisher Scientific, Massachusetts) and resuspended in 100 µl diethyl ether. As described in (LaFountain *et al.*, 2015), the samples were subjected to saponification prior to analysis: 100 µl of 5% ethanolic KOH were added, and the samples were vortexed and incubated for 2h in the dark at 21 °C. After addition of 400 µl hexane : diethyl ether (1:1), samples were washed with 500 µl H_2O and placed on ice for phase separation. Washing of samples was repeated ca. 4-5 times until the pH of the aqueous phase was neutral on pH-paper. The upper phase was transferred to a vitreous UHPLC vial (Infochroma, Switzerland) and evaporated in the SpeedVac concentrator. Samples were re-solubilised in 60 µl acetonitrile : MeOH : H_2O (87:10:3) and analysed on a C18 Hypersil ODS column (250 × 4.6 mm; Thermo Electron) at 25°C using the following gradient of solvent A (20% 1M ammonium acetate, 80% MeOH) and B (20% acetone, 80% MeOH): 0-15 min, 100% B; 15-25 min, 100% B; 25-28 min, 0% B; 28-32min, 0% B. The flow rate was set up to 1 mL min^{-1} and 40 µL of each sample was injected. Parameters were controlled by a Gynkotek liquid chromatography system (Thermo Fisher Scientific, Massachussetts) equipped with a UVD340S diode array detector set at 450 nm for carotenoids identification. The two main carotenoids β-carotene and lycopene were identified and quantified as described for the anthocyanins using calibration curves of authentic reference standards.

Transcriptome assembly

mRNA-Seq was performed for one individual per morph from Puflatsch, one individual from Ofenpass, Switzerland, two individuals from Chandolin, Switzerland, one *G. densiflora* plant from each of two populations near Tschierv, Switzerland and Davos Dorf, Switzerland, one *G. odoratissima* plant from a population near Munstertal, Switzerland, and one *G. conopsea* plant from a population near Cinuos-chel-Brail, Switzerland. RNA was extracted from the remaining 50% of the ground flowers used for UHPLC-MS/MS (see above). Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific, Massachusetts) according to the manufacturer's protocol and subsequently purified using a Qiagen RNeasy MinElute cleanup kit (Qiagen, Netherlands). RNA was quantified on a 2100 Bioanalyzer (Agilent Technologies, California) and sequenced paired-end on one lane of an Illumina HiSeq 2500 platform (Illumina, California). mRNA-seq reads were trimmed using *Trimmomatic* v. 0.36 (Bolger *et al.*, 2014) to remove Illumina adapters, leading and trailing bases below quality 3, truncate reads when a 4-bp sliding window average quality dropped below 15, and finally discard reads below 36bp in length. Surviving reads were then *de-novo* assembled to transcripts using *Trinity* v. 2.0.6 (Grabherr *et al.*, 2011).

Generation of mRNA expression profiles

Expression profiling was conducted for 45 plants from Puflatsch, for twenty-six plants from Chandolin, and for six plants from Ofenpass. Total RNA was extracted from the other half of the ground flower tissue used for UHPLC-MS/MS (see above) using TRIzol reagent (Thermo Fisher Scientific, Massachusetts) according to the manufacturer's protocol. Samples were quantified with a Qubit fluorometer and RNA HS assay kit (Thermo Fisher Scientific, Massachusetts), and libraries were prepared with a Lexogen QuantSeq 3' mRNA-Seq kit (Lexogen, Austria) according to the manufacturer's protocol. All libraries were pooled and sequenced single-end on one lane of an Illumina HiSeq 2500 platform (Illumina, California).

Differential expression analysis

Raw expression profile reads were trimmed to a minimal length of 20 bp with *Trimmomatic* v. 0.36 (Bolger *et al.*, 2014). Trimmed reads were then mapped to the reference transcriptome, and expression levels were estimated using *RSEM* v. 1.2.31 (Li & Dewey, 2011) and *bowtie2* v. 2.2.9 (Langmead & Salzberg, 2012) as aligner. Posterior mean counts per morph were subsequently extracted with a custom *R* script. Transcripts with a total of less than 6 read counts in less than 2 samples were removed, and negative binomial generalized log-linear models were fitted for transcripts with an at least two-fold expression difference using *edgeR* v. 3.12.1 (Robinson *et al.*, 2010). Transcripts with a significant expression difference were searched against the NCBI nucleotide database with blastn (Altschul *et al.*, 1990).

Transcriptome-wide association study (TWAS)

Trimmed expression profile reads (see above) were mapped to the indexed reference transcriptome with *bowtie2* v. 2.2.9 (Langmead & Salzberg, 2012) and converted to indexed bam files. Variant calling of these files was performed with *samtools* v. 1.3 (Li *et al.*, 2009) excluding bases with a quality of less than 13. The output was indexed and converted to vcf format using *bcftools* v. 0.1.20 (Li *et al.*, 2009). Subsequently, conversion from vcf to hapmap format was performed in *R* v.3.2.4, using the *biOP4R* library function bdVcf2Hapmap (https://sourceforge.net/projects/biop) under exclusion of variants with a minimal depth of less than 15. Normalised spectral reflectance at 517 nm wavelength was extracted for each sample from the measured spectral curves (see above), and transcriptome-wide association of variants with the spectral data was assessed by fitting compressed mixed linear models with *GAPIT* v 2 (Lipka *et al.*, 2012), including the first seven principal components. Sequences of the ten transcripts with the strongest association were searched against the NCBI nucleotide database with blastn (Altschul *et al.*, 1990).

Phylogenetic analysis

Coding sequences of *Arabidopsis thaliana MYB1-16, 32, 75, 90, 111, 113, 114,* and *123* and *Phalaenopsis equestris MYB1-14,* and *16* were retrieved from GenBank and aligned with *GrMYB1* using *MEGA* v. 7 (Tamura *et al.*, 2007). The sequence of the conserved *R2R3* domain was extracted and used for phylogenetic analysis with *MrBayes* v. 3.2.6 as described in (Streisfeld & Rausher, 2008).

Genotyping

Genotyping was conducted for 88 plants from Puflatsch, 23 plants from Monte Bondone, 16 plants from Reschenpass (all Italy), 5 plants each of populations from Chandolin, Furkapass, Berninapass, Davos, and Ofenpass (all Switzerland), Rifflsee, Astental, and Heiligenbachalm, and 4 plants from Stubalpe (all Austria, Tables S1 and S2). Total RNA was extracted with TRIzol reagent as described above, and 1µg of RNA was treated with DNAse I (Thermo Fisher Scientific, Massachusetts) according to the manufacturer's protocol. The DNAse I-treated RNA was converted to cDNA using Revert-aid H^- Reverse Transcriptase (Thermo Fisher Scientific, Massachusetts) according to the manufacturer's protocol. Full *GrMYB1* coding sequence was amplified with Phusion HotStart II polymerase (Thermo Fisher Scientific, Massachusetts); PCR: 4 µl HF buffer (5×), 0.4 µl dNTPs (10 mM), 2 µl *GrMYB1* - F / F2 primer (5 µM, Table S10), 2 µl *GrMYB1* - R primer (5 µM, Table S10), 0.2 µl Revert-aid H^-, 1 µl cDNA, 10.4 µl ddH_2O; PCR-conditions: 98°C for 30 s, 35 cycles of (98°C for 10 s, 62.6°C for 30 s, 72°C for 30 s), 72°C for 5 min. For cleanup, 5 µl of PCR-reaction was mixed with 0.5 µl of Exo I (20U / µl), and 2 µl FastAP (1U / µl, both Thermo Fisher Scientific, Massachusetts), and incubated at 37°C for 20 min, and at 85°C for 15 min. Clean PCR-reactions were Sanger-sequenced on an Applied Biosystems 3130xl genetic analyser using

BigDye™ Terminator v3.1 Cycle Sequencing chemistry (Thermo Fisher Scientific, Massachusetts); Sequencing PCR: 2 µl sequencing buffer (5×), 1 µl *GrMYB1* - F / F2 / R primer (5 µM, Table S10), 1 µl BigDye™ v. 3.1, 1µl clean PCR-reaction, 5 µl ddH_2O; PCR conditions: 96°C for 2 min, 30 cycles of (96°C for 10 s, 50°C for 5 s, 60°C for 4 min). For plants with no or degraded RNA available, DNA was extracted from leaf tissue dried in silica gel using a Qiagen DNEasy plant mini kit (Qiagen, Netherlands) according to the manufacturer's protocol, and a 385bp fragment in the last coding exon was amplified and sequenced as explained above. Relative peak heights / fluorescence intensities of each nucleotide at the SNP positions were extracted from the .ab1 trace files using a custom *R* script. Nucleotides with a relative fluorescence intensity of ≥20% were counted as present.

Production of *GrMYB1* dsRNA

Full coding sequence of the last exon of the wild-type *GrMYB1* allele was amplified with Phusion HotStart II polymerase (Thermo Fisher Scientific, Massachusetts); PCR: 4 µl HF buffer (5×), 0.4 µl dNTPs (10 mM), 2 µl *GrMYB1* - attB - F primer (5 µM, Table S10), 2 µl *GrMYB1* - attB - R primer (5 µM, Table S10), 0.2 µl Revert-aid H^-, 1 µl cDNA, 10.4 µl ddH_2O; PCR-conditions: 98°C for 30 s, 5 cycles of (98°C for 10 s, 65°C for 30 s, 72°C for 40 s), 35 cycles of (98°C for 10 s, 72°C for 40 s), 72°C for 5 min. PCR-reactions were cleaned up with a NucleoSpin® Extract II kit (Macherey-Nagel, Germany) according to the manufacturer's protocol. The amplicons were cloned into a *p*DONR207 donor vector using GATEWAY™ cloning (Thermo Fisher Scientific, Massachusetts): 75 ng of cleaned PCR fragment was combined with 75 ng *p*DONR207 and 1 µl BP-clonase II, and ddH_2O was added to 5 µl. Reactions were incubated at 25°C for 2 h, 0.5 µl Proteinase K (2 µg/µl) was added, and reactions were incubated at 37°C for 10 min. Chemo-competent *E. coli* DH5α (Thermo Fisher Scientific, Massachusetts) were transformed with this construct using a standard protocol, and plasmids were extracted with a QIAprep® Spin Miniprep Kit (Qiagen, Netherlands) according to the manufacturer's protocol. In a second step, the *GrMYB1* fragment was cloned into a *p*L4440 destination vector, a gift from Andrew Fire (Addgene plasmid # 1654): 50 ng of *p*DONR207-construct was combined with 75 ng *p*L4440, 1 µl LR-clonase II, and ddH_2O was added to 5 µl. The reaction was incubated as described above, and chemo-competent *E. coli* HT115 (courtesy Anita Dirks) were transformed with this construct using a standard protocol. dsRNA was produced according to (Lau *et al.*, 2014) with some modifications: After overnight incubation at 37°C / 220 rpm in 5 ml LB medium with 50 µg/ml ampicillin and 10 µg/ml Tetracycline, the culture was dissolved 1:100 in 500 ml 2× YT medium with the same concentration of antibiotics, and incubated at 37°C / 220 rpm. At OD_{600} = 0.7, 2 ml 0.1 M Isopropyl-*β*-D-thiogalactopyranosid (IPTG) was added to a final dilution of 0.4 mM, and the culture was incubated for another 2h at 37°C / 220 rpm. After

centrifugation for 10 min at 3200 × *g*, the supernatant was removed, and the pellet was resuspended in 10 ml 0.1 M phosphate buffer (pH7.2; 720 µl 1M Na_2HPO_4, 280 µl 1M NaH_2PO_4, 9 ml H_2O). Cells were disrupted in a Potter-Elvehjem homogenisator, and by freeze-thawing the suspension five times in liquid N_2. After centrifugation for 20 min at 3200 × *g*, the supernatant was collected, filtered through Miracloth (Merck, Germany), and stored at -80°C.

RNA-interference (RNAi)

Whole, expanding *G. rhellicani* inflorescences were cut at Ofenpass (Table S1) on 17 July 2017 and transferred to the lab in water-filled vases. The dsRNA-solution was mixed in a petridish with carborundum (400 mesh, Sigma Aldrich, Switzerland), and twenty-two inflorescences were inoculated by rubbing one side of the inflorescences in the solution, leaving the other side of the inflorescence untreated. Inflorescences were kept at 21°C/60% relative humidity/150 µmol $m^{-2}S^{-1}$ light intensity at day (8:00 to 20:00), and 12°C/60% relative humidity/no light at night. After 7 days, five flowers each from two treated plants showing a phenotype and four mock-inoculated control plants were collected in liquid N_2. Total RNA was extracted from these flowers, and 1 µg of RNA was DNAse I treated as described above. DNAse I-treated RNA was converted to cDNA using the double amount of reagents as described above, and split into two aliquots with and without the addition of reverse transcriptase. Reverse-transcription quantitative real-time PCR (RT-qPCR) was performed in triplicates on a CFX96 Touch™ Real-Time PCR Detection System (BioRad Laboratories, California) using Maxima SYBR green (Thermo Fisher Scientific, Massachusetts); PCR: 1.2 µl Primer - F (5 µM, Table S10), 1.2 µl Primer - R (5 µM, Table S10), 0.5 µl ROX (0.25 µM), 10 µl Maxima SYBR Green Master Mix, 1 µl cDNA, 6.1 µl ddH_2O; PCR-conditions: 95°C for 10 min, 40 cycles of (95°C for 15s, 60°C for 30 s, 72°C / plate readout for 30 s). The three reference genes *glyceraldehyde 3-phosphate dehydrogenase* (*G3PDH*), *Oligopeptidase* (*OPTD*), and *4-α-glucanotransferase* (*4-α-GTF*) were chosen based on the literature (Schlüter *et al.*, 2011; Monteiro *et al.*, 2012) and also showed low variation in expression between samples of the expression profile dataset (see above). For each control and treatment plant, the quantification cycle (C_q) was averaged over all three replicates per reference gene. The average C_q of all four reference genes was then subtracted from the average C_q of the target genes to obtain ΔC_q. 95% confidence intervals were calculated for the ΔC_q values of the four control plants and compared to the ΔC_q values of the treatment plants.

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Supplementary Figures

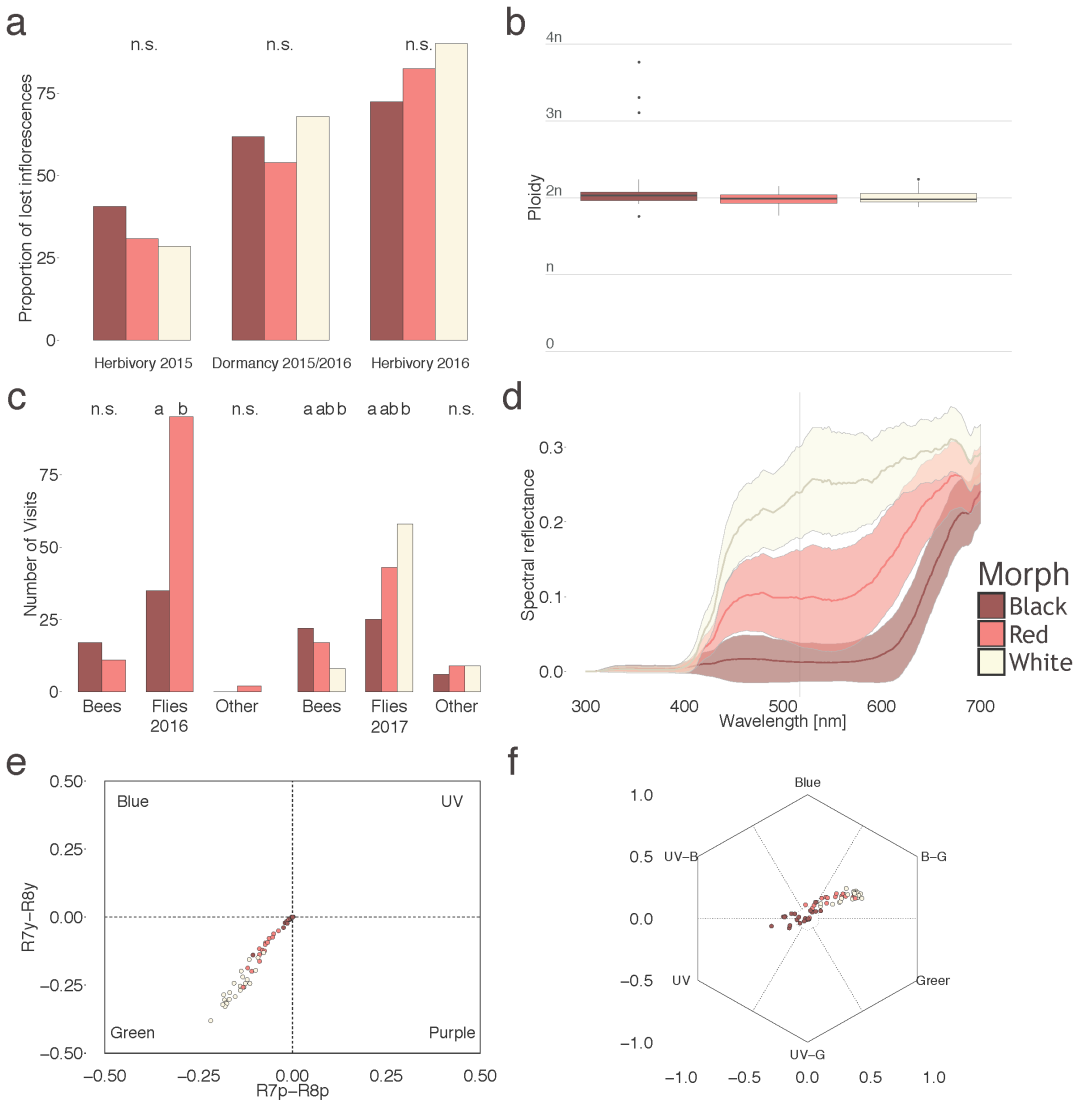

Figure S1 | Ecological underpinnings of the colour polymorphism in *G. rhellicani* a, Loss of plants due to herbivory by insects and livestock (high in 2016 due to browsing by horses instead of cows, chi χ^2-test (n=281), χ^2=1.473, *P*=0.479), as well as after winter dormancy is not different between colour morphs (χ^2-test (n=281), χ^2=0.544, *P*=0.762). **b,** Apart from three single polyploid individuals, flow cytometry identified all plants as diploid. **c,** Time lapse video recordings of 2016 (χ^2-test bees (n=28), χ^2=1.286, *P*=0.257, χ^2-test flies (n=130), χ^2=27.692, *P*=1.422 × 10^{-7}, χ^2-test others (n=2), χ^2=2.000, *P*=0.157) and 2017 (χ^2-test bees (n=47), χ^2=6.426, *P*=0.040, χ^2-test flies (n=126), χ^2=13.000, *P*=0.002, χ^2-test others (n=24), χ^2=0.750, *P*=0.687) show that bees preferentially visit dark plants, while flies prefer bright plants. **d,** Mean ± 1sd of the spectral reflectance of the three colour morphs from 300 to 700 nm. The vertical line denotes the absorbance maximum of the two main cyanidin pigments at 517 nm. **e,** Spectral reflectance of the colour morphs maps to the green quadrant of the fly visual space, with black plants positioned in the achromatic centre. **f,** Bees perceive *G. rhellicani* flowers as blue-green with black and a part of the red plants in the achromatic centre.

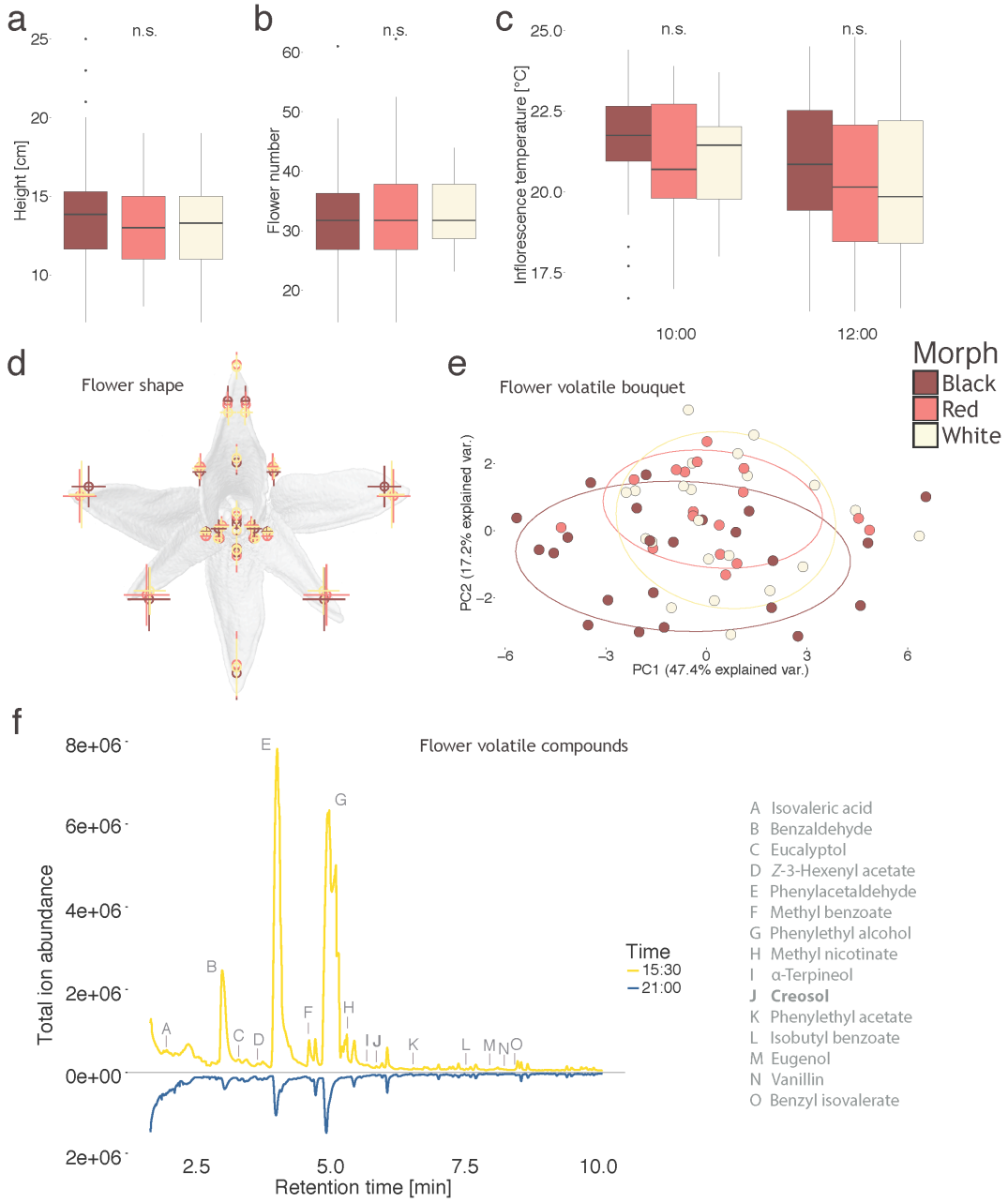

Figure S2 | Differences in pollinator-relevant phenotypic traits between the *G. rhellicani* colour morphs a, Inflorescence height is not different between *G. rhellicani* colour morphs (ANOVA (n=343), $F(2, 340)=1.341$, $P=0.263$). **b,** Flower number does also not differ between colour morphs (ANOVA (n=271), $F(2, 268)=0.342$, $P=0.710$). **c,** Black plants do not heat up more than red or white morphs under solar radiation (10:00: ANOVA (n=60), $F(2, 57)=0.328$, $P=0.722$, 12:00: ANOVA (n=90), $F(2, 87)=0.437$, $P=0.648$). **d,** Flower shape is not significantly different between colour morphs (procrustes ANOVA of symmetrical component (n=48), $F(2, 45)=0.708$, $P=0.732$, procrustes ANOVA of asymmetrical component (n=48), $F(2, 45)=1.191$, $P=0.294$). The graphic shows the mean symmetrical procrustes coordinates ± 1 sd per morph. **e,** Principle component analysis showed no separation of colour morphs according to floral volatile bouquet. **f,** Floral volatile analysis identified 15 compounds contributing ≥ 1% to the floral bouquet, of which only creosol showed a significantly reduced emission in red and white morphs (non-labelled peaks are contaminants also detected in the air

control samples). After sunset, scent emission is strongly reduced in all morphs (blue curve). The well-known chocolate-like odour of *G. rhellicani* can be attributed to the presence of vanillin (peak N).

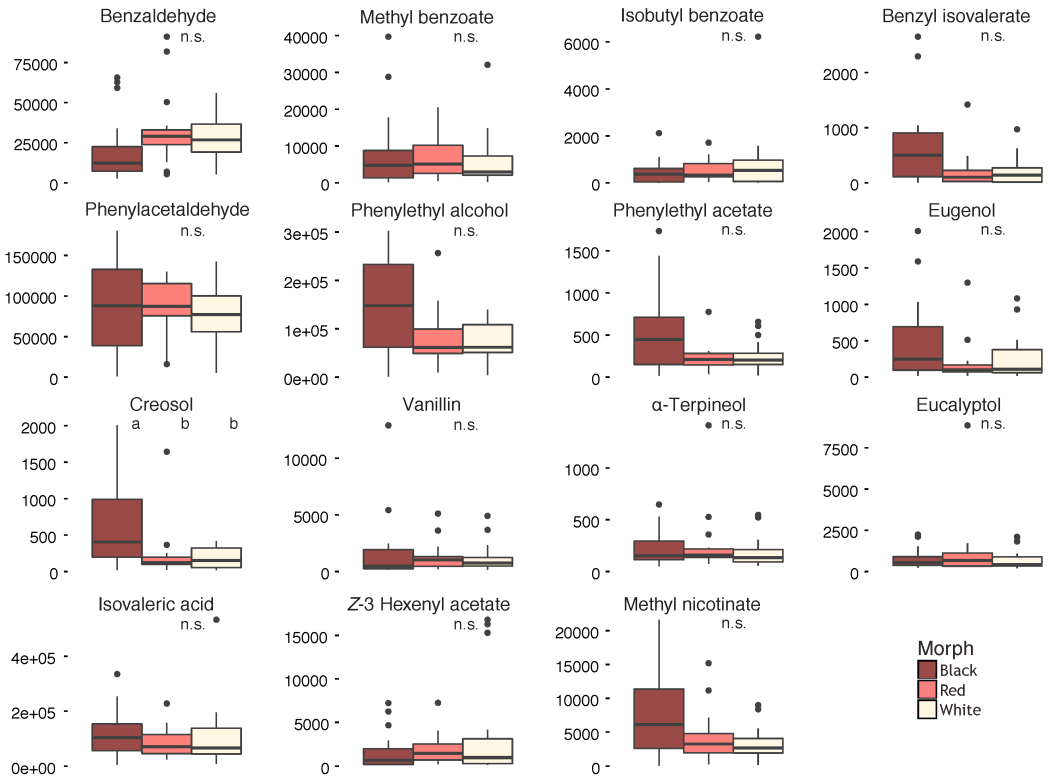

Figure S3 | VOC quantities in the floral scent bouquet of the three *G. rhellicani* colour morphs Emission rates in pg per l air of the 15 VOC which account for > 1% of the total floral scent bouquet recorded in the Puflatsch population. Apart from a trend towards a reduced emission of aromatic compounds in red and white morphs (only statistically significant for the minor compound Creosol) there was no difference in VOC composition between the three colour morphs (See also Table S2).

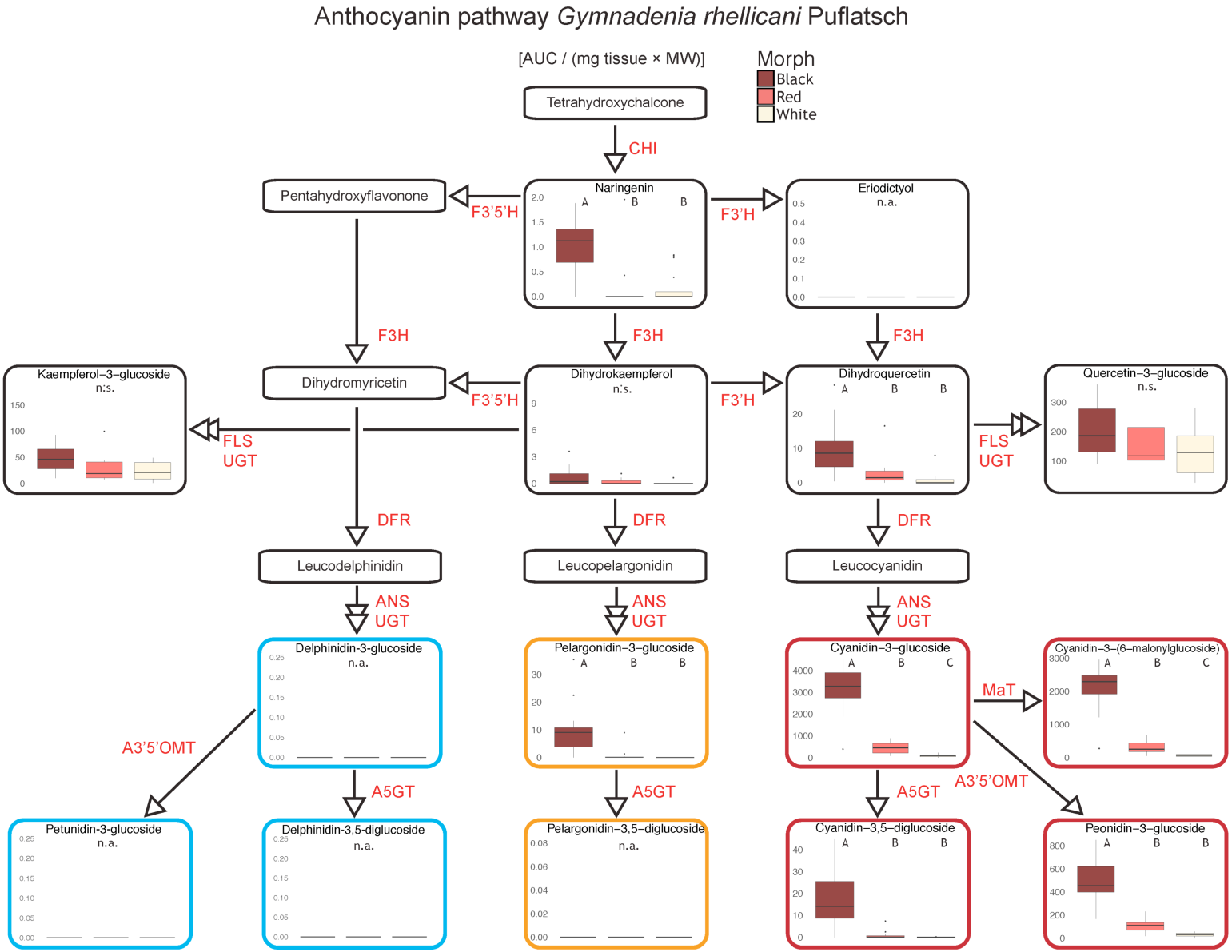
Anthocyanin pathway *Gymnadenia rhellicani* Puflatsch
[AUC / (mg tissue × MW)]
Morph
Black
Red
White
Tetrahydroxychalcone
CHI
Naringenin
Pentahydroxyflavonone
Eriodictyol
n.a.
F3'5'H
F3'H
F3H
Kaempferol–3–glucoside
n.s.
Dihydromyricetin
Dihydrokaempferol
n.s.
Dihydroquercetin
Quercetin–3–glucoside
n.s.
FLS
UGT
DFR
Leucodelphinidin
Leucopelargonidin
Leucocyanidin
ANS
UGT
Delphinidin-3-glucoside
n.a.
Pelargonidin–3–glucoside
Cyanidin–3–glucoside
Cyanidin–3–(6–malonylglucoside)
MaT
A3'5'OMT
A5GT
Petunidin-3-glucoside
n.a.
Delphinidin-3,5-diglucoside
n.a.
Pelargonidin–3,5–diglucoside
n.a.
Cyanidin–3,5–diglucoside
Peonidin–3–glucoside

Figure S4 | UHPLC-MSMS quantifications of anthocyanins and precursors in flowers of the three *G. rhellicani* colour morphs The anthocyanin pathway is divided in three branches producing blue delphinidin, orange pelargonidin, and red cyanidin pigments. Quantification of anthocyanins (area under curve (AUC) / mg tissue × molecular weight (MW), coloured frames) and precursors (black frames) showed that only the cyanidin branch is fully active in *G. rhellicani*, hence the red colour of the plants. Also, further modifications *e.g.* the addition of different sugar residues and chemical groups, can slightly change the hue of an anthocyanin compound. The red colour in *G. rhellicani* flowers is due to cyanidin-3-glucoside, its derivative cyanidin-3-(6-malonylglucoside), and to a lesser degree, another derivative peonidin-3-glucoside. The amount of the two main cyanidins is reduced more than 7.5 × in red, and more than 30 × in white colour morphs (ANOVA and TukeyHSD *post hoc* test, see Table S4). All compounds were identified and quantified using authentic reference standards, except for cyanidin-3-(6-malonylglucoside), which was identified by an interpretation of the data spectrum obtained and based on previous results (see methods section). Standards for the two delphinidin precursors pentahydroxyflavonone and dihydromyricetin were unavailable, and leucodelphinidin, -pelargonidin, and -cyanidin are unstable intermediates. Enzyme abbreviations: CHI: chalcone isomerase, F3'5'H: flavonoid 3',5'-hydroxylase, F3'H: flavonoid 3'-hydroxylase, F3H: flavonoid 3-hydroxylase, FLS: flavonol synthase, UGT: UDP-glucose 3-O-glucosyltransferase, DFR: dihydroflavonol 4-reductase, ANS: anthocyanidin synthase, A3'5'OMT: A3',5'-O-methyltransferase, A5GT: anthocyanin 5-O-glucosyltransferase, MaT: malonyl transferase.

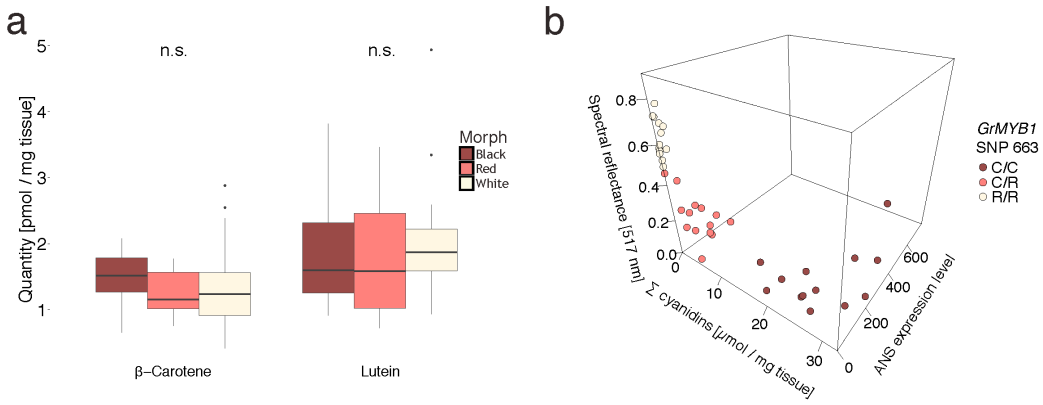

Figure S5 | Further analyses of *G. rhellicani* flower pigmentation a, Concentration of the two main floral carotenoids, β-carotene and lutein, is not different between the colour morphs (β-carotene: ANOVA (n=43), $F(2, 40)=0.520$, $P=0.598$, lutein: ANOVA (n=45), $F(2, 42)=0.614$, $P=0.546$). **b,** Correlation between phenotype (spectral reflectance at 517 nm), metabolomics (combined amount of the two main cyanidins), and transcriptomics (*ANS* expression level). Samples are coloured according to genotype (SNP at position 663 in the *GrMYB1* transcript, R = A or G).

Supplementary Tables

Table S1 | Coordinates of the populations assessed in this study Table showing the latitude and longitude coordinates in WGS 84-format, as well as the elevation in meters above sea level (m.a.s.l.) of the assessed *G. rhellicani* populations.

Population	Latitude	Longitude	Elevation [m.a.s.l.]
Chandolin	46° 15' 11.3" N	07° 36' 39.2" E	2290
Furkapass	46° 35' 14.0" N	08° 26' 10.2" E	2255
Albulapass	46° 34' 53.2" N	09° 48' 50.2" E	2210
Davos	46° 48' 39.9" N	09° 49' 20.9" E	1980
Berninapass	46° 26' 43.5" N	09° 58' 33.4" E	2080
Ofenpass	46° 38' 02.4" N	10° 17' 00.5" E	2295
Reschenpass	46° 49' 13.9" N	10° 32' 44.9" E	2270
Rifflsee	46° 58' 03.9" N	10° 50' 10.3" E	2255
Bondone	46° 00' 25.8" N	11° 01' 39.7" E	1695
Puflatsch	46° 33' 22.4" N	11° 36' 37.6" E	2110
Sajatkopf	47° 01' 57.0'' N	12° 22' 20.0'' E	2320
Edelweißspitze	47° 07' 23.0'' N	12° 49' 30.0'' E	2305
Astental	46° 57' 35.7'' N	12° 57' 26.4'' E	2055
Heiligenbachalm	46° 56' 34.3'' N	13° 44' 35.2'' E	1925
Stubalpe	47° 05' 08.0'' N	14° 56' 05.0'' E	1635

Table S2 | Sample sizes of the experiments conducted in this study Table showing the number of black, red, and white *G. rhellicani* plants per population, which were used in the experiments of this study. Analyses marked with an asterisk (*) included the same 48 focal plants from Puflatsch (or a subset thereof).

Experiment	Puflatsch			Bondone			Other	Total
	Black	Red	White	Black	Red	White	Black	
Fitness	175	78	28	0	0	0	263	544
Flow cytometry*	23	14	20	4	13	0	179	253
Pollinator exclusion	0	0	0	0	0	0	40	40
Pollinator recordings 2016	11	11	0	0	0	0	0	22
Pollinator recordings 2017	10	10	10	0	0	0	0	30
Spectral mapping*	23	15	26	7	24	1	153	249
Height*	198	91	54	7	24	1	406	781
Temperature	50	50	50	0	0	0	0	150
3D morphometrics*	16	16	16	0	0	0	101	149
Floral volatile analysis*	23	15	24	7	24	1	155	249
Floral volatile time course	7	0	7	0	0	0	7	21
UHPLC anthocyanins*	16	14	17	7	24	1	189	268
HPLC carotenoids*	15	14	16	7	24	1	30	107
Transcriptome assembly*	1	1	1	0	0	0	3	6
mRNA expression profiles*	14	14	15	0	0	0	33	76
Differential expression*	14	14	14	0	0	0	0	42
TWAS*	14	14	15	0	0	0	0	43
Genotyping*	31	26	31	7	15	1	75	186
RNAi	0	0	0	0	0	0	24	24

Table S3 | Insect taxa recorded on *G. rhellicani* inflorescences at Puflatsch Insects were identified down to family, and if possible, to genus or species level. Numbers in brackets indicate insects with pollinia attached. The 2015 dataset was compiled by catching insects observed on *G. rhellicani* inflorescences along east-west transects on both ridges. Numbers of recordings may thus be biased by insect visibility and ability to escape catching. The 2016 and 2017 datasets are based on the time-lapse camera recordings and should thus be unbiased.

Collection 2015

Order	Family	Species	# on Black	# on Red	# on White
Coleoptera	Scarabaeidae	unknown	1 (0)	0	0
Coleoptera	Mycteridae	unknown	0	1 (0)	0
Diptera	Anthomyiidae	unknown	3 (0)	2 (0)	2 (0)
Diptera	Empididae	unknown	1 (0)	0	1 (0)
Diptera	Muscidae	unknown	11 (1)	6 (1)	4 (0)
Diptera	Rhagonidae	unknown	1 (0)	0	0
Diptera	unknown	unknown	3 (0)	5 (1)	3 (1)
Hymenoptera	Apidae	Apis mellifera	0	0	1 (0)
Hymenoptera	Apidae	Bombus sp.	5 (1)	2 (0)	1 (0)
Lepidoptera	Crambidae	unknown	16 (3)	6 (0)	3 (1)
Lepidoptera	Noctuidae	Autographa sp.	1 (0)	0	0
Lepidoptera	Noctuidae	unknown	1 (1)	0	0
Lepidoptera	Nymphalidae	Coenonympha gardetta	0	1 (1)	1 (0)
Lepidoptera	Nymphalidae	Coenonympha pamphilus	0	0	1 (1)
Lepidoptera	Nymphalidae	Erebia sp.	1 (0)	0	0
Lepidoptera	Tortricidae	unknown	3 (0)	3 (1)	1 (0)
Lepidoptera	Zygaenidae	Zygaena sp.	4 (1)	0	0
Lepidoptera	unknown	unknown	0	3 (0)	0

Table S4 | ANOVA tables from the linear mixed-effects models of floral VOC emission in *G. rhellicani* colour morphs Prior to analysis, all VOC data was BoxCox transformed to approach normality. Differences in VOC emission between the colour morphs were modelled using holm adjusted linear mixed-effects models with sampling year as random factor (n=62), and Tukey HSD *post-hoc* tests. F (2,58): conditional F-statistic, *P*-adj: holm adjusted *P*-value (*P*<0.05 in bold), *post-hoc*: summary of TukeyHSD *post-hoc* comparisons (B: black, R: red, W: white).

Compound	F (2,58)	*P*-adj	*post-hoc*
Benzaldehyde	4.507	0.212	n.a.
Methyl benzoate	0.128	1.000	n.a.
Isobutyl benzoate	1.956	1.000	n.a.
Benzyl isovalerate	3.223	0.561	n.a.
Phenylacetaldehyde	0.159	1.000	n.a.
Phenylethyl alcohol	4.055	0.292	n.a.
Phenylethyl acetate	2.456	0.946	n.a.
Eugenol	2.439	0.946	n.a.
Creosol	8.126	**0.012**	$t_{B\text{-}R}$ = -3.122, $P_{B\text{-}R}$ = **0.005** $t_{B\text{-}W}$ = -3.693, $P_{B\text{-}W}$ **< 0.001** $t_{R\text{-}W}$ = -0.318**,** $P_{R\text{-}W}$ = 0.946
Vanillin	0.117	1.000	n.a.
α-Terpineol	0.552	1.000	n.a.
Eucalyptol	0.500	1.000	n.a.
Isovaleric acid	0.342	1.000	n.a.
Z-3 Hexenyl acetate	0.574	1.000	n.a.
Methyl nicotinate	3.032	0.615	n.a.

Table S5 | Flavonoids identified in *G. rhellicani* flowers Table showing the physical and chemical properties of all flavonoids identified in this study. Naringenin, dihydrokaempferol, and pelargonidin-3-glucoside were present at low intensity, and eriodictyol, pelargonidin-3,5-diglucoside, and delphinidin compounds were not detected. EC: elemental composition, RT: retention time (min), m/z: observed mass by charge, Mass: theoretical mass [M]+/[M+H]+, MSMS: tandem mass spectrometry fragments (n.f.: no fragmentation under the present experimental conditions), λ_{max}: absorbance peaks in the UV/VIS spectrum.

Compound	**EC**	**RT**	**m/z**	**Mass**	**MSMS**	**λ_{max}**
Naringenin	$C_{15}H_{12}O_5$	7.90	273.0761	273.0758	**273**-> 153, 147, 119	low
Kaempferol 3-glucoside	$C_{21}H_{20}O_{11}$	6.22	449.1080	449.1078	**449**-> 287	264, 346
Dihydrokaempferol	$C_{15}H_{10}O_6$	8.16	287.0554	287.0550	n.f.	low
Quercetin 3-glucoside	$C_{21}H_{20}O_{12}$	5.96	465.1036	465.1028	**465**-> 303	256, 352
Dihydroquercetin	$C_{15}H_{10}O_7$	7.42	303.0499	303.0499	n.f.	254, 370
Pelargonidin 3-glucoside	$C_{21}H_{21}O_{10}$	4.97	433.1140	433.1129	**433**-> 271	low
Cyanidin 3-glucoside	$C_{21}H_{21}O_{11}$	4.73	449.1073	449.1078	**449**-> 287	208,280,516
Cyanidin 3,5-diglucoside	$C_{27}H_{31}O_{16}$	4.18	611.1609	611.1607	**611**-> 449, 287	276, 518
Peonidin 3-glucoside	$C_{22}H_{23}O_{11}$	5.01	463.1200	463.1235	**463**-> 301	276, 516
Cyanidin 3-(6-malonylglucoside)	$C_{24}H_{23}O_{14}$	5.10	535.1087	535.1082	**535**-> 287	280,516

Table S6 | ANOVA tables of flavonoid quantities in *G. rhellicani* colour morphs Relative quantities of anthocyanins and precursor compounds were calculated from the UHPLC-MSMS chromatograms as area under curve (AUC) / mg tissue × molecular weight (MW), and BoxCox transformed to approach normality. Differences in anthocyanin metabolite quantities between the colour morphs were modelled using holm adjusted ANOVAs (n=47), and Tukey HSD *post-hoc* tests. Delphinidin derivatives, Eriodictyol, and Pelargonidin-3,5-diglucoside were not present in the samples and were thus excluded from the analysis. F (2,44): conditional F-statistic, *P*-adj: holm adjusted *P*-value (*P*<0.05 in bold), *post-hoc*: summary of TukeyHSD *post-hoc* comparisons (B: black, R: red, W: white).

Compound	**F (2,44)**	***P*-adj**	***post-hoc***
Naringenin	14.118	**9.17×10^{-5}**	$t_{B\text{-}R}$ = -4.603, $P_{B\text{-}R}$ **< 0.001** $t_{B\text{-}W}$ = -4.575, $P_{B\text{-}W}$ **< 0.001** $t_{R\text{-}W}$ = 0.102**,** $P_{R\text{-}W}$ = 0.994
Eriodictyol	n.a.	n.a.	n.a.
Kaempferol-3-glucoside	4.016	0.075	n.a.
Dihydrokaempferol	1.117	0.336	n.a.
Quercetin-3-glucoside	3.689	0.075	n.a.
Dihydroquercetin	12.213	**2.42×10^{-4}**	$t_{B\text{-}R}$ = -3.610, $P_{B\text{-}R}$ **= 0.002** $t_{B\text{-}W}$ = -4.715, $P_{B\text{-}W}$ **< 0.001** $t_{R\text{-}W}$ = -1.028**,** $P_{R\text{-}W}$ = 0.564
Delphinidin-3-glucoside	n.a.	n.a.	n.a.
Delphinidin-3,5-diglucoside	n.a.	n.a.	n.a.
Petunidin-3-glucoside	n.a.	n.a.	n.a.
Pelargonidin-3-glucoside	12.213	**1.75×10^{-5}**	$t_{B\text{-}R}$ = -4.808, $P_{B\text{-}R}$ **< 0.001** $t_{B\text{-}W}$ = -5.306, $P_{B\text{-}W}$ **< 0.001** $t_{R\text{-}W}$ = -0.412**,** $P_{R\text{-}W}$ = 0.911
Pelargonidin-3,5-diglucoside	n.a.	n.a.	n.a.
Cyanidin-3-glucoside	97.900	**5.86×10^{-16}**	$t_{B\text{-}R}$ = -7.387, $P_{B\text{-}R}$ **< 0.001** $t_{B\text{-}W}$ = -13.981, $P_{B\text{-}W}$ **< 0.001** $t_{R\text{-}W}$ = -6.366**,** $P_{R\text{-}W}$ **< 0.001**
Cyanidin-3,5-diglucoside	28.687	**7.42×10^{-8}**	$t_{B\text{-}R}$ = -6.313, $P_{B\text{-}R}$ **< 0.001** $t_{B\text{-}W}$ = -6.750, $P_{B\text{-}W}$ **< 0.001** $t_{R\text{-}W}$ = -0.328**,** $P_{R\text{-}W}$ = 0.943
Peonidin-3-glucoside	76.207	**4.07×10^{-14}**	$t_{B\text{-}R}$ = -9.473, $P_{B\text{-}R}$ **< 0.001** $t_{B\text{-}W}$ = -11.552, $P_{B\text{-}W}$ **< 0.001** $t_{R\text{-}W}$ = -1.891**,** $P_{R\text{-}W}$ = 0.153
Cyanidin-3-(6-malonylglucoside)	98.299	**5.86×10^{-16}**	$t_{B\text{-}R}$ = -7.422, $P_{B\text{-}R}$ **< 0.001** $t_{B\text{-}W}$ = -14.008, $P_{B\text{-}W}$ **< 0.001** $t_{R\text{-}W}$ = -6.358**,** $P_{R\text{-}W}$ **< 0.001**

Table S7 | Transcripts differentially expressed between black and white *G. rhellicani* colour morphs The transcript most differentially expressed between black and white *G. rhellicani* colour morphs maps to *anthocyanidin synthase* (*ANS*) genes. Analysis with *edgeR* v. 3.12.1 (n=42); logFC: absolute log-fold changes between average expression levels per morph, usr-logFC: Un-shrunk log-fold expression level changes between morphs, logCPM: average log counts per million, *P*-adj: *P*-value adjusted with False Discovery Rate (FDR) correction (*P*<0.05 in bold).

Transcript	BLASTn	logFC	usr-logFC	logCPM	*P*-adj
TR261401.c0_g1	*anthocyanidin synthase* (*ANS*)	4.156	4.170	6.903	**3.25×10^{-4}**
TR112617.c0_g3	no hit	3.903	3.960	4.476	**3.25×10^{-4}**
TR224389.c1_g2	no hit	4.366	4.376	7.316	**0.007**
TR97685.c0_g2	no hit	5.886	7.496	2.620	**0.032**
TR262779.c6_g4	no hit	3.379	3.427	4.535	**0.032**
TR200635.c1_g9	no hit	4.924	5.171	3.735	**0.032**
TR234590.c1_g6	no hit	3.781	4.853	1.894	**0.032**
TR202234.c2_g5	no hit	3.308	3.348	4.460	**0.033**
TR218853.c1_g6	*peptidyl-prolyl cis-trans isomerase*	3.644	3.889	2.841	**0.033**

Table S8 | Top ten SNPs associated with spectral reflectance at λ_{517} of the *G. rhellicani* colour morphs Three of the top ten SNPs associated with spectral reflectance at 517 nm wavelength, the absorption maximum of cyanidins, are situated in the 3'-UTR of the same *R2R3-MYB* transcription factor. Analysis with GAPIT v. 2 (n=43); MAF: Frequency of the minor allele, R^2-exc: R^2 of the model excluding the SNP, R^2-inc: R^2 of the model including the SNP, *P*: (unadjusted) *P*-value.

Transcript	Position	BLASTn	MAF	R^2-exc	R^2-inc	*P*
TR259745.c4_g1	177	no hit	0.233	0.397	0.694	**2.08×10^{-4}**
TR224083.c24_g2	59	uncharacterized	0.477	0.397	0.672	**3.28×10^{-4}**
TR272909.c1_g9	777	uncharacterized	0.477	0.397	0.612	**1.21×10^{-3}**
TR101194.c0_g1	1623	no hit	0.430	0.397	0.604	**1.44×10^{-3}**
TR255984.c3_g4	375	*snRNP*	0.453	0.397	0.603	**1.46×10^{-3}**
TR255984.c3_g4	376	*snRNP*	0.453	0.397	0.603	**1.46×10^{-3}**
TR237780.c2_g1	968	*R2R3-MYB*	0.465	0.397	0.601	**1.54×10^{-3}**
TR237780.c2_g1	978	*R2R3-MYB*	0.453	0.397	0.591	**1.96×10^{-3}**
TR237780.c2_g1	886	*R2R3-MYB*	0.465	0.397	0.590	**1.98×10^{-3}**
TR263130.c2_g1	155	no hit	0.256	0.397	0.589	**2.03×10^{-3}**

Table S9 | Mean ΔC_q values and confidence intervals of *GrMYB1* and *GrANS1* in *G. rhellicani* inflorescences after RNAi with *GrMYB1* Table showing the mean quantification cycle difference (ΔC_q) and the 95% confidence interval (CI) for *GrMYB1* and *GrANS1* in the four control, and the two treated *G. rhellicani* inflorescences. The mean ΔC_q of the RNAi-treated plant, which showed a strong reduction of cyanidins in the petals (in bold), lies clearly outside the 95% confidence interval of the mean of the controls (oblique) for both *GrMYB1* and *GrANS1.*

Plant	*GrMYB1*		*GrANS1*	
	ΔC_q [mean ± 1SD]	**CI [α=0.95]**	**ΔC_q [mean ± 1SD]**	**CI [α=0.95]**
Control 1	0.26 ± 2.27	-4.19, 4.70	-4.24 ± 0.34	-4.90, -3.57
Control 2	2.20 ± 0.24	1.74, 2.67	-1.03 ± 0.21	-1.44, -0.62
Control 3	1.11 ± 0.21	0.69, 1.53	-3.50 ± 0.17	-3.83, -3.16
Control 4	1.83 ± 0.28	1.29, 2.37	-1.33 ± 0.23	-1.78, -0.88
Mean controls	1.35 ± 1.15	*0.70, 2.00*	-2.52 ± 0.25	*-2.66, -2.38*
Treatment 1	**3.42** ± 0.35	2.73, 4.11	**-0.05** ± 0.26	-0.57, 0.47
Treatment 2	0.62 ± 0.60	-0.55, 1.78	-2.80 ± 0.61	-3.99, -1.60

Table S10 | Primer sequences used in this study Table showing primer sequences used for genotyping, cloning, and RT-qPCR

Primer sequences used for genotyping	
GrMYB1 - F	5' ATGGAGAGAGAGAGGGGGG
GrMYB1 - R	5' TCATTGTTCCCAATCAACATC
GrMYB1 - F2 (last exon)	5' TAATGCATGGACAGCAACAG
Primer sequences used for cloning (attB-sites in capital)	
GrMYB1 - attB - F	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTAAtaatgcatggacagcaacag
GrMYB1 - attB - R	5' GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAtcattgttcccaatcaacatcaag
Primer sequences used for qRT-PCR	
GrMYB1 - qPCR - F	5' CGACCCGACGATATTAATGC
GrMYB1 - qPCR - R	5' CGGATCAAAGGATTCAAAGC
GrANS1 - qPCR - F	5' GCGAATTGAACGGGAGAAG
GrANS1 - qPCR - R	5' AAGATTGCCCAAGTTCAAGC
GrG3PDH - qPCR - F	5' TCCTCAGGATTTCTAACCCCAAAG
GrG3PDH - qPCR - R	5' CTTCATCACCACCGAATACATGAC
GrOPTD - qPCR - F	5' GAGCTGCACTTTCTACATCTGATG
GrOPTD - qPCR - R	5' CACTTGTAGCATCCCCAAAGAAAC
Gr4αGTF - qPCR - F	5' CTTCTTGTGTCCATCCTGTTATGC
Gr4αGTF - qPCR - R	5' GCTCACTTGGCATTCTCTGAATAC

General Discussion

Interest in the field of insect-mediated plant microevolution is as old as biology itself, and each era has left its legacy of theories and hypotheses characterized by its predominant perspectives and techniques. Scientific advance not only requires a constant re-evaluation of these hypotheses, they also have to be re-integrated into a steadily growing evolutionary framework of increasing complexity. The two hypotheses evaluated in this work have been challenging to amalgamate with the contemporary understanding of microevolution, mainly because they have yet to pass the currently undergoing fusion of partially diametric research fields: Overdominance was coined as a clean, mathematical model to explain phenomena occurring under the controlled conditions of plant breeding, and the population genetics theories to which overdominance was later transferred by Dobzhansky were no less abstract. As a rule, situations in nature are much more complex and ecological settings often create a spatiotemporal mosaic of different micro-evolutionary processes. While it can be quite straightforward to detect mechanisms eroding or enhancing phenotypic variation under these circumstances, finding evidence for balancing processes conferring stability of variation may be much more challenging. One of the tasks of eco-evo-devo research will thus be to find ways of disentangling these ties to be able to empirically test the importance of classic theoretical hypotheses such as overdominance in natural populations. The situation for epigenetic effects is not much different. Over many years, the concept of genetic inheritance and Mendelian segregation provided clear and simple boundaries for the research of most biologists, with notable exceptions such as Barbara McClintock's study on transposable elements (McClintock, 1950). The idea that some of the multi-layered and fuzzy developmental settings can also be heritable and thus have to be integrated in the micro-evolutionary framework has thus stirred up long-concluded discussions.

The results of the work presented herein indicate that epigenetic mechanisms are involved in a bi-directional fine-tuning of plant-insect interactions. Insect herbivory can significantly alter a plant methylome, which may be connected to changes in plant phenotypic traits (chapter I). To close the circle, induced phenotypic alterations again influence the behaviour of insect visitors such as pollinators and parasitoids (chapter I and II). Changes in this chain of causation can thereby persist for more than one generation after treatment, excluding the action of potentially confounding cytoplasmic maternal effects (chapter II). It is important to note that while herbivory seems to affect a great number of epiloci and induces changes in a wide range of traits (chapter I and II), only a fraction of trait changes is retained more than one generation after treatment (chapter II). Also, the residual changes are rather linked to resource limitation than to adaptive changes in plant communication with pollinators and parasitoids. Two deductions can be made from these findings: Firstly, this work

provides no clear evidence of Lamarckian evolution, which is often attributed to epigenetic processes. Herbivores may appear all of a sudden, but their presence is usually ephemeral. Epigenetic changes are thus an ideal mechanism to exploit the norm of reaction to spontaneous environmental changes within a few plant generations. However, a long-term retention of epigenetic changes providing resource-consuming protection against a long-gone herbivore would obviously be maladaptive, in line with the observed lack of inheritance of acquired, initially adaptive epimutations. Secondly, the general importance of single epimutations in long-term micro-, or even macro-evolutionary adaptive processes is questionable. Theoretically, other biotic and abiotic environmental factors could induce a similarly profound perturbance of the epigenome with a similarly unspecific inheritance pattern as the herbivore species in this work. Under such prerequisites, the continuous change in environmental conditions of many habitats would create a rapid turnover of epimutations. In a macro-evolutionary timespan, this epigenetic turnover would then rather appear as undirected developmental noise shrouding long-term genetic transitions. Nevertheless, genetics and epigenetics are interlinked, and the fitness of an organism is ultimately determined by the different epigenomes a genome can produce. Since it is currently challenging to separate the genetic and epigenetic components of a phenotype, epigenetic inheritance was tested in a setting with minimized genetic variation (chapter II). Following studies should thus aim to account for genetic-epigenetic covariation, and once technical advance permits, study the role of epigenetics also in microevolution of natural populations.

Results from the genetic part of this work most likely provide the hitherto clearest evidence for a role of overdominance in insect-mediated plant microevolution. The applied eco-evo-devo approach allowed addressing all of Hedrick's prerequisites for a demonstration of overdominance in the focal colour polymorphic *Gymnadenia rhellicani* population on Alm Puflatsch, namely identification of the genes and alleles under selection, the relative fitness of each genotype, and the mechanism of selection (Hedrick, 2012). Moreover, the comprehensive understanding of the *G. rhellicani* system enables the formulation of hypotheses on the prerequisites for the emergence of insect-mediated overdominance in natural populations: Firstly and most simply, *G. rhellicani* is diploid and neither autogamous nor apomictic, a conceptual basis for this mode of selection. Secondly, the causative mutation is located in an *R2R3-MYB* transcription factor, a highly diverse gene family, which has undergone extensive duplication, sub-, and neo-functionalization (Feller *et al.*, 2011). Also, the affected structural gene regulated by this transcription factor, *ANS,* is located towards the end of the anthocyanin pigment pathway. Under these circumstances, negative and positive pleiotropic effects are expected to be relatively limited, which reduces the chance that the polymorphism may be under associative overdominance. Thirdly, the alleles at the locus under selection produce two phenotypic states and are codominant. Although both polymorphic *G. rhellicani*

populations on Alm Puflatsch and Monte Bondone have three alleles each at their corresponding loci, in both cases, two of these alleles have the same effect on the protein, resulting in three graded phenotypes per population (However, the selective forces at Monte Bondone are yet unexplored). Fourthly, the polymorphic trait is highly fitness relevant, and the homozygous mutated phenotype overshoots the fitness maximum. In the present case, the superior fitness of the heterozygote is a cumulative effect of opposite directional selection of bee and fly pollinators. However, a simpler scenario with a single pollinator is in principle also conceivable. Fifthly, in contrast to many other polymorphic systems, *G. rhellicani* is a rewarding, nectar producing plant, speaking against the presence of other, oscillating types of balancing selection based on the avoidance of common morphs by pollinators. These factors may help in identification of other, yet unknown overdominant systems and ultimately contribute to an estimation of the prevalence of this mode of selection in nature. However, the ultimate individual prerequisites for the emergence of overdominance may only be understood if the evolutionary history of the polymorphic organism is uncovered. For *G. rhellicani,* this knowledge is still fragmentary, yet advanced enough to allow the formulation of a first hypothesis: The genus *Gymnadenia* encompasses two groups of plants with contrasting morphology. While one group of species is characterized by tall inflorescences with ample spaced, resupinated (rotated) flowers in light-toned red to violet hues, the group including *G. rhellicani,* formerly known under the genus *Nigritella,* has short, strongly condensed inflorescences with unresupinated, intensely coloured flowers. There is compelling evidence that the *Nigritella* section is derived and represents a paedomorphic state of a tall *Gymnadenia* plant (Box & Glover, 2010). The intense, black-red coloration of *G. rhellicani* might thus be the result of a constraint in pigmentation, i.e. the inability of *G. rhellicani* to reduce pigment production in parallel to the spatial condensation of flower tissue. This constraint was then later relaxed in multiple independent ways: Firstly, the *Nigritella* section contains several taxa of higher ploidy and less dark coloration that *G. rhellicani*, so polyploidization might have played a role in escaping the constraint. Nevertheless, the polyploid *Gymnadenia* species are most likely completely autogamous, and the ecological consequences of the colour shifts remain to be assessed. Secondly, another species from the *Nigritella* section, *G. miniata,* is diploid and has bright red flower coloration. The evolutionary history, the genetic background, and the ecology of *G. miniata* are still unexplored. Thirdly, the amount of pigments was reduced through loss of function mutations as represented in the two polymorphic *G. rhellicani* populations. Overdominance may thus have conferred a swing-back of *G. rhellicani* to an ancestral, optimal coloration of medium intensity. However, the mutations in the two populations are different, and the two polymorphisms must thus have evolved independently. This raises the question whether these populations share particular prerequisites for the relaxation of this constraint in coloration. Since

the second polymorphic population on Monte Bondone is still insufficiently studied, hypotheses are rather speculative and include a possible similarity in pollinator assemblage, climatic conditions (both populations are on the lower end of the altitudinal range of *G. rhellicani*), soil composition (Alm Puflatsch is situated on a volcanic outcrop), or genetic predispositions of *G. rhellicani* populations in the Southern Alps. As a side note, it should also be mentioned that the morphology of white *G. rhellicani* inflorescences is strikingly similar to the sympatric alpine clover *Trifolium alpinum*, which is highly abundant at the Monte Bondone site. It can thus not be excluded that Müllerian mimicry also plays a role in this population. Future investigations on the mode of selection at Monte Bondone as well as phylogenetic analyses of different *G. rhellicani* populations combined with environmental recordings might thus help identifying the factors that allow a species to vary and retain this variation under balancing selection.

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Curriculum Vitae

Name	KELLENBERGER
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Education

Sep 2002 - Sep 2008	High school, Kantonsschule Zürcher Unterland, Bülach (Altsprachliches Profil) Matura thesis: "Spectral characterization and mapping of *Welwitschia mirabilis* in Namibia"
Sep 2008 - Sep 2011	Bachelor of Science in Environmental Sciences, University of Zurich. Bachelor thesis: "Germination and establishment of Dipterocarp seedlings in logged lowland rainforest, Sabah, Borneo", supervised by Dr. Michael O'Brien and Prof. Dr. Andrew Hector.
Sep 2008 - Sep 2011	Bachelor of Science in Biology, University of Zurich (UZH)
Dec 2011 - Dec 2012	MSc student in Evolutionary Biology, Department of Systematic and Evolutionary Botany, University of Zurich, Switzerland. Master thesis: „Construction of a virus-induced gene silencing system for European and tropical orchids", supervised by Dr. Philipp Schlüter
Feb 2013 - current	Ph.D. in Biology, Department of Systematic and Evolutionary Botany, University of Zurich, Switzerland. PhD subproject 1: "Eco-evo-devo of a floral colour polymorphism in the Alpine orchid *Gymnadenia* (*Nigritella*) *rhellicani*", supervised by Dr. Philipp Schlüter PhD subproject 2: "Causes and consequences of epigenetic variation in plant interactions with pollinators and herbivores", University Research Priority project (URPP) supervised by Prof. Dr. Florian Schiestl.

Student supervision and teaching activities

Jun 2015 - present — Co-instruction of PhD students at the Institute of Systematic and Evolutionary Botany, University of Zurich

- Srignanakshi Iyer, "Molecular basis of parallel evolutionary divergence by differential pollinator attraction"
- Laura Piñeiro " The ecological genomics of parallel adaptation and speciation"

Sep 2013 - Sep 2015 — Teaching assistance at the Institute of Systematic and Evolutionary Botany, University of Zurich

- BIO 221 "Plant-Insect Interactions" (supervision of 3 student projects)
- BIO 113 "Evolution", University of Zurich (assistance in practical parts)

Scientific reviewing activities

Aug 2017 — Invited reviewer for Molecular Genetics and Genomics
Aug 2016 — Invited reviewer for Molecular Ecology

Competitive research grants

Apr 2018 - Sep 2019 — SNF Early Postdoc.Mobility grant, Zurich (CHF 77650)
Feb 2017 - Dec 2017 — Forschungskredit Candoc, University of Zurich (CHF 52750)
Jun 2017 - Sep 2017 — G. & A. Claraz Foundation travel grant, Zurich (CHF 958)
Jun 2015 - Sep 2015 — G. & A. Claraz Foundation travel grant, Zurich (CHF 935)

Participation in conferences and talks

Jan 2017 — "Dobzhansky reloaded: Eco-evo-devo of an overdominant colour polymorphism in the Alpine orchid *Gymnadenia* (*Nigritella*) *rhellicani"* Plant and Animal Genomics (PAG) symposium, **San Diego**, CA (talk)

Nov 2016 — "Evolution of a colour polymorphism in *Gymnadenia rhellicani* (Orchidaceae)" International Symposium on Pollination Biology, **Vienna**, Austria (poster)

Aug 2015 — "(Epi)genetics and ecology of floral colour polymorphisms in the alpine orchid *Gymnadenia* (=*Nigritella*) *rhellicani"* European Society of Evolutionary Biology (ESEB) conference, **Lausanne**, Switzerland (poster)

Dec 2015 "A floral colour polymorphism in *Gymnadenia rhellicani* (Orchidaceae)" Plant Science Centre (PSC) symposium, **Zurich**, Switzerland (poster)

Sep 2013 "Causes and consequences of epigenetic variation in plant interactions with pollinators and herbivores" PSC-Syngenta symposium, **Stein**, Switzerland (talk)

Aug 2009 "Spectral characterization and mapping of *Welwitschia mirabilis* in Namibia" IEEE International Geoscience and Remote Sensing Symposium, **Cape Town**, ZA (poster)

Public outreach

Jan 2017 Article "*Welwitschia mirabilis*: Ein Dinosaurier der Botanik" for the society "Freunde des Botanischen Gartens Zürich", also published on UZH facebook and twitter pages

Sep 2015 Organisation of a PhD symposium of the Institute of Systematic and Evolutionary Botany, University of Zurich

Sep 2014 "Carnivorous plants" Seminar at the botanical garden, University of Zurich, Switzerland (public lecture)

Nov 2013 "*Welwitschia mirabilis*" Seminar at the botanical garden, University of Zurich, Switzerland (public lecture)

Awards

Aug 2011 Swiss Botanical Society: Certificate in Field Botany Level 1 with award

Apr 2009 43. Swiss National Contest, Schweizer Jugend Forscht: predicate "outstanding" Special prize "Albrecht von Haller"

June 2008 The 50 best graduation papers of the year, Impuls Mittelschule: special prize

Aug 2008 Geographic-Ethnographic Society Zurich: GEGZ promotional award

Publications

Kellenberger RT, Byers KJRP, De Brito Francisco RM, Staedler YM, LaFountain AM, Schönenberger J, Schiestl FP, Schlüter PM. Emergence of a floral colour polymorphism by pollinator-mediated overdominance. **In preparation**

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O'Brien MJ, Burslem DFRP, **Kellenberger RT**, Philipson CD, Tay J, Ong R, Reynolds G, Hector A. Temporal dynamics of seedling recruitment in a logged forest after a large-scale mast fruiting. **Submitted to Journal of Ecology**

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